The molecular basis of differences in the receptor-binding properties of antigenically distinct influenza haemagglutinins.

Thesis

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The Molecular Basis of Differences in the Receptor-Binding Properties of Antigenically Distinct Influenza Haemagglutinins

A Thesis submitted to the Open University for the Degree of Doctor of Philosophy

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Abstract

The uptake of influenza viruses into host cells is initiated by binding of their haemagglutinins (HA) to terminal sialic acids on receptors. After infection, the immune system produces anti-HA antibodies that block receptor-binding activity and thereby neutralise virus infectivity. As a consequence, antigenic variation is necessary for a new influenza epidemic to occur. Since many epitopes are close to the receptor-binding site (RBS) of HA, it was the aim of this thesis to investigate whether changes in antigenicity correlated with differences in receptor-binding properties.

A microscale-assay using surface plasmon resonance (SPR) was developed to study the interaction of virus particles with receptor analogues. This technique provides information on kinetic rate constants and affinities for biomolecular interactions. The conditions of the assay were established using bovine fetuin, which contains sialic acid in the α(2,3)- and α(2,6)-linkage to terminal galactose of oligosaccharides. Viruses isolated from different hosts vary in their ability to recognise sialic acid in these linkages. Therefore, the assay was performed using fetuins derivatised to contain sialic acid either in the α(2,3)- or the α(2,6)-linkage, in order to determine the linkage-specificity of influenza viruses.

Various antigenically distinct H1 and H3 subtype viruses, chosen on the basis of reactivity with post-infection ferret sera, were tested for their receptor-binding properties. It was shown that antigenic evolution of HA for both subtypes was associated with changes in affinities for receptor analogues. Sequencing of the gene coding for HA revealed that the changes correlated with amino acid substitutions close to or in the RBS. The importance of the variant residues for the observed changes in receptor-binding properties is discussed using the previously solved X-ray structures of HAs complexed

with receptor analogues.

Finally, the molecular basis for the evolution of avian H3 HAs to recognise sialic acid in the α(2,6)-linkage characteristic of human HAs was studied by *in vitro* selection of receptor-binding variant viruses. Although selection of an avian HA with a substitution at residue 226 previously reported by Rogers et al. (1985) was not achieved, a variant with a substitution at residue 201 was identified.

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Abbreviations

mAb	monoclonal antibody
APS	ammonium persulphate
BHA	bromelain-released HA
BHK	baby hamster kidney
BSA	bovine serum albumin
CAM	chorio-allantoic membranes
CMP-Neu5Ac	cytidine-5'-monophospho-N-acetylneuraminic acid
Da	daltons
DMEM	Dulbecco's modified eagle's medium containing pyridoxine
cDNA	complementary DNA
EDTA	ethylenediaminetetraacetic acid
eggs	embryonated hens' eggs
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmatic reticulum
FCS	fetal calf serum
Gal	galactose
GalNAc	N-acetyl-galactosamine
GlcNAc	N-acetyl-glucosamine
HA	haemagglutinin
HAU	haemagglutination unit
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HI	haemagglutination inhibition
HRP	horseradish peroxidase

Ig	immunoglobulin
k_a	association rate constant
k_d	dissociation rate constant
K_D	equilibrium dissociation constant
k_m	mass transport rate
k_{obs}	observed rate constant
LAIV	live attenuated influenza virus
M1	matrix 1
M2	matrix 2
MDCK	Madin-Darby canine kidney cells
MES	2-morpholinoethanesulfonic acid
min	minutes
MW	molecular weight
MWCO	molecular weight cut-off
NA	neuraminidase
NAI	neuraminidase activity inhibition
Neu5Ac	N-acetylneuraminic acid
Neu5Gc	N-glycolylneuraminic acid
NIMR	National Institute for Medical Research
NMR	nuclear magnetic resonance
NP	nucleocapsid protein
dNTP	deoxynucleotidetrisphosphates
PAUP	Phylogenetic Analysis Using Parsimony
PBS	phosphate-buffered saline
PCR	poylmerase chain reaction

RBC	red blood cells
RBS	receptor-binding site
RDE	receptor-destroying enzyme
mRNA	messenger RNA
ssRNA	single-stranded RNA
vRNA	viral RNA
RNP	ribonucleoprotein
RT-PCR	reverse transcriptase-poylmerase chain reaction
RU	resonance unit
SA	streptavidin
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
sec	seconds
SI	specificity index
SL	sialyllactose
SLN	sialyllactosamine
SPR	surface plasmon resonance
TBE	Tris-Borate/EDTA
TEMED	N,N,N',N'-Tetramethylethylenediamine
TLC	thin-layer chromatography
TPCK	L-(tosylamido-2-phenyl) ethyl chloromethyl ketone
Tris	Tris-Hydroxymethylaminoethane
VERO	green monkey kidney
WHO	World Health Organisation
WIC	World Influenza Centre

1 Introduction

HA plays a major role in influenza virus cell entry and immune response. Studies of antigenic variation and receptor-binding properties of HA will therefore help in understanding the continued evolution of influenza viruses in the human host.

1.1 Discovery of the Influenza Virus

Based on clinical observation and epidemiology, influenza-like disease outbreaks have been reported since the 12th century. However, influenza virus as the causative agent was not isolated until 1933. Driven by an influenza wave in the London area, scientists at the National Institute for Medical Research (NIMR) decided to make use of their canine distemper study facilities to identify and culture the virus. Therefore, ferrets were inoculated with filtered throat washings of infected colleagues. The animals developed influenza-like illness, transmitted the disease to other ferrets and were rendered immune to re-infection (Smith et al., 1933). Finally, the first virus was isolated from one of the scientists called Wilson Smith and thus named after him. Serial passage of the virus in ferrets resulted in a highly pathogenic strain for mice, and finally Koch's postulates were fulfilled by accident, when a ferret infected with a mouse-adapted influenza virus sneezed at a scientist. He developed flu-like symptoms and the virus isolated from his throat washings proved to be the same virus as given to the ferret (Smith and Stuart-Harris, 1936).

1.2 Classification

The name influenza (italian for "influence") is based on the old medical belief in unfavourable astrological *influences* as the cause of the disease. Influenza viruses belong

to the *Orthomyxoviridae* (greek *orthos* for "standard, correct", and *myxo* for "mucus") family of enveloped viruses with a segmented genome of single-stranded RNA of negative polarity. They are classified into three genera: Influenza A, B and C, based on differences of their nucleocapsid protein (NP) and matrix 1 (M1) protein antigens (WHO, 1953; Schild, 1972). Influenza A viruses infect a variety of mammals and birds, whereas Influenza B and C have only been isolated from humans, with the exception of a number of type C isolates from pigs (Guo et al., 1983) and a type B isolate from seals (Osterhaus et al., 2000). Influenza C contains 7 genome segments, whereas Influenza A and B contain 8 segments. Influenza A viruses are further divided into subtypes based on the antigenic nature of their HA and neuraminidase (NA) glycoproteins. To date 16 HA (H1-H16) and 9 NA (N1-N9) subtypes have been identified (Schild et al., 1980; Fouchier et al., 2005). Whereas all 16 HA subtypes circulate in aquatic birds, 3 have evolved in humans (H1, H2, H3), at least 2 in pigs (H1, H3) and 2 in horses (H3, H7) (Webster et al., 1992).

1.3 The Influenza Virus Particle

1.3.1 Structure

The Influenza A virus particle contains 8 single-stranded RNA (ssRNA) segments of negative polarity (McGeoch et al., 1976) coding for 10 viral proteins (see Figure 1a). Each of these is independently associated with NP to form a coiled hairpin structure (Pons et al., 1969; Compans et al., 1972; Jennings et al., 1983), with the polymerase complex proteins (PB1, PB2 and PA) attached to the 5' and 3' ends of the RNA (Hagen et al., 1994). The complex of viral RNA (vRNA), NP and the polymerase proteins forms the ribonucleoprotein (RNP) particle. The virion is enveloped by a host-derived lipid bilayer (Kates et al., 1961) containing the glycoproteins HA and NA. HA is the more abundant of the two, a virion containing 300-400 HA in contrast to approximately 50 NA molecules

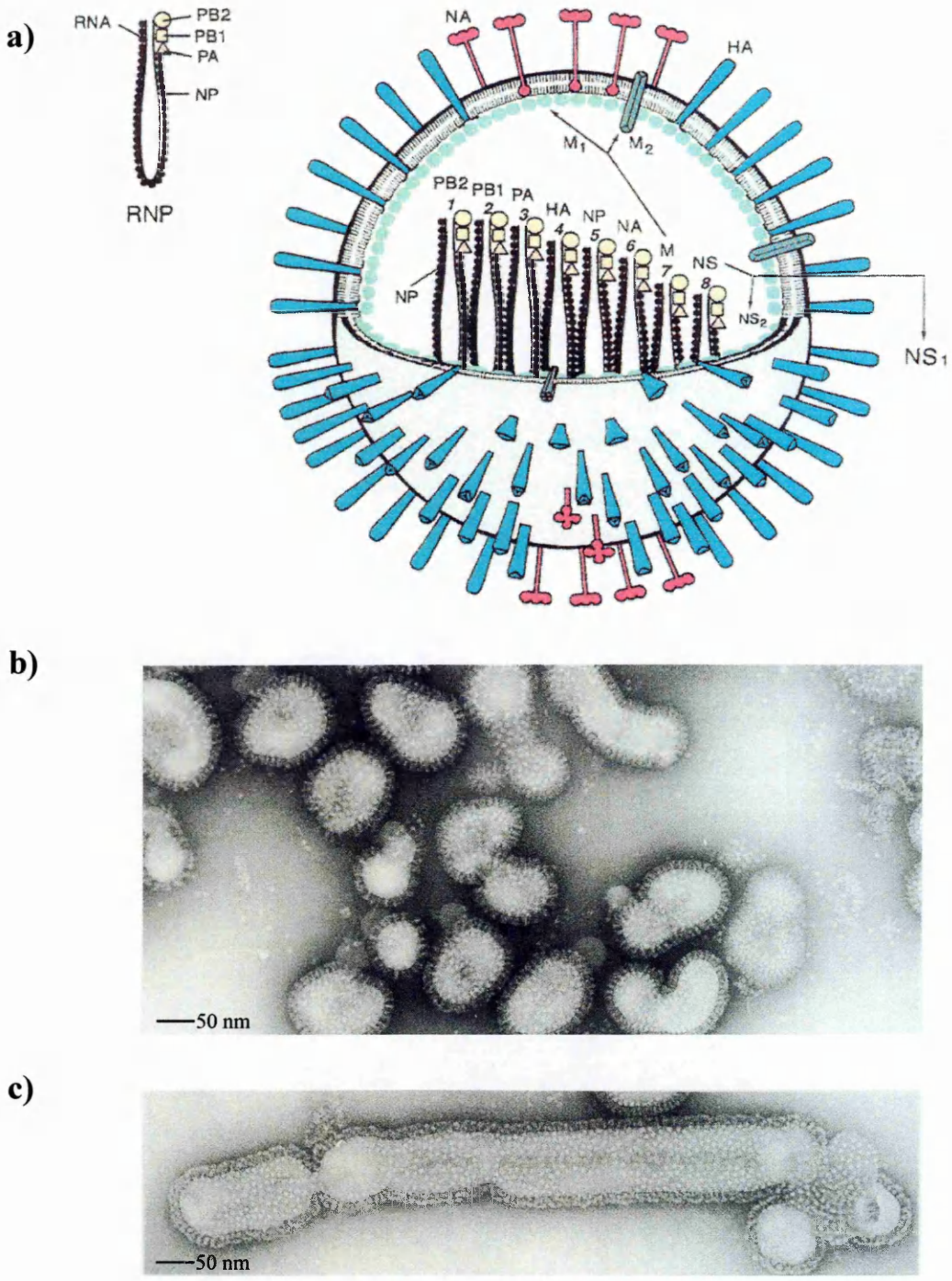


Figure 1 Structure of the influenza A virus

a) Schematic diagram of the influenza virus particle (adapted from Wright and Webster, 2001a). The surface of the virus particle contains 3 integral membrane proteins: haemagglutinin (HA), neuraminidase (NA) and M2 channel protein. Matrix protein (M1) underlies the lipid membrane. The 8 viral RNA segments are each associated with the 3 subunits of the polymerase complex (PB1, PB2 and PA) and are encapsidated by nucleoprotein (NP), forming the ribonucleoproteins (RNPs). The other virally encoded proteins include NS1 and NS2 (NEP). **b)** Negative-stain electron micrograph of spherical virus (X31, H3N2) **c)** Negative-stain electron micrograph of filamentous virus (Chita/1/03, H3N2) Figures **b)** and **c)** were taken by L. Calder (NIMR, London).

(Ruigrok et al., 1984; Ruigrok, 1998). A few copies (~ 14-68) of a third transmembrane protein are embedded in the envelope, the membrane-channel matrix 2 (M2) protein (Zebedee and Lamb, 1988; Sugrue and Hay, 1991). Underlying the lipid bilayer is a shell of M1, which is the major structural component of the virus (Schulze, 1970; Schulze, 1972).

1.3.2 Morphology

Electron microscope studies reveal that influenza virions are pleomorphic, ranging from small spherical particles with a diameter of 80-120 nm (Taylor et al., 1943) to long filaments up to several μm in length (Mosley and Wyckoff, 1946) (see Figure 1b and c). HA and NA appear as spikes protruding from the virus envelope. Clinical human and animal isolates are predominantly filamentous (Chu et al., 1949), and a gradual shift from filamentous to spherical morphology has been observed upon serial passages in embryonated hens' eggs (eggs) (Burnet and Lind, 1957; Choppin et al., 1960). Other morphologies such as "kidney"-shape and "double-kidney"-shape are artefacts of sample preparation for negative-staining electron microscopy and reinforce the impression of variability of the virus (Nermut and Frank, 1971; Ruigrok and Hewat, 1991).

1.4 The Influenza Virus Life Cycle

An overview of the virus life cycle is presented in Figure 2.

Influenza viruses bind to cells by interaction of HA with sialylated receptors (reviewed in Gottschalk, 1959), leading to receptor-mediated endocytosis (Fazekas de St. Groth, 1948b; Matlin et al., 1981; Sieczkarski and Whittaker, 2002). Acidification of the endosome triggers a conformational change in the HA structure, resulting in fusion of the viral and endosomal membranes (White et al., 1981; Skehel et al., 1982; Bullough et al.,

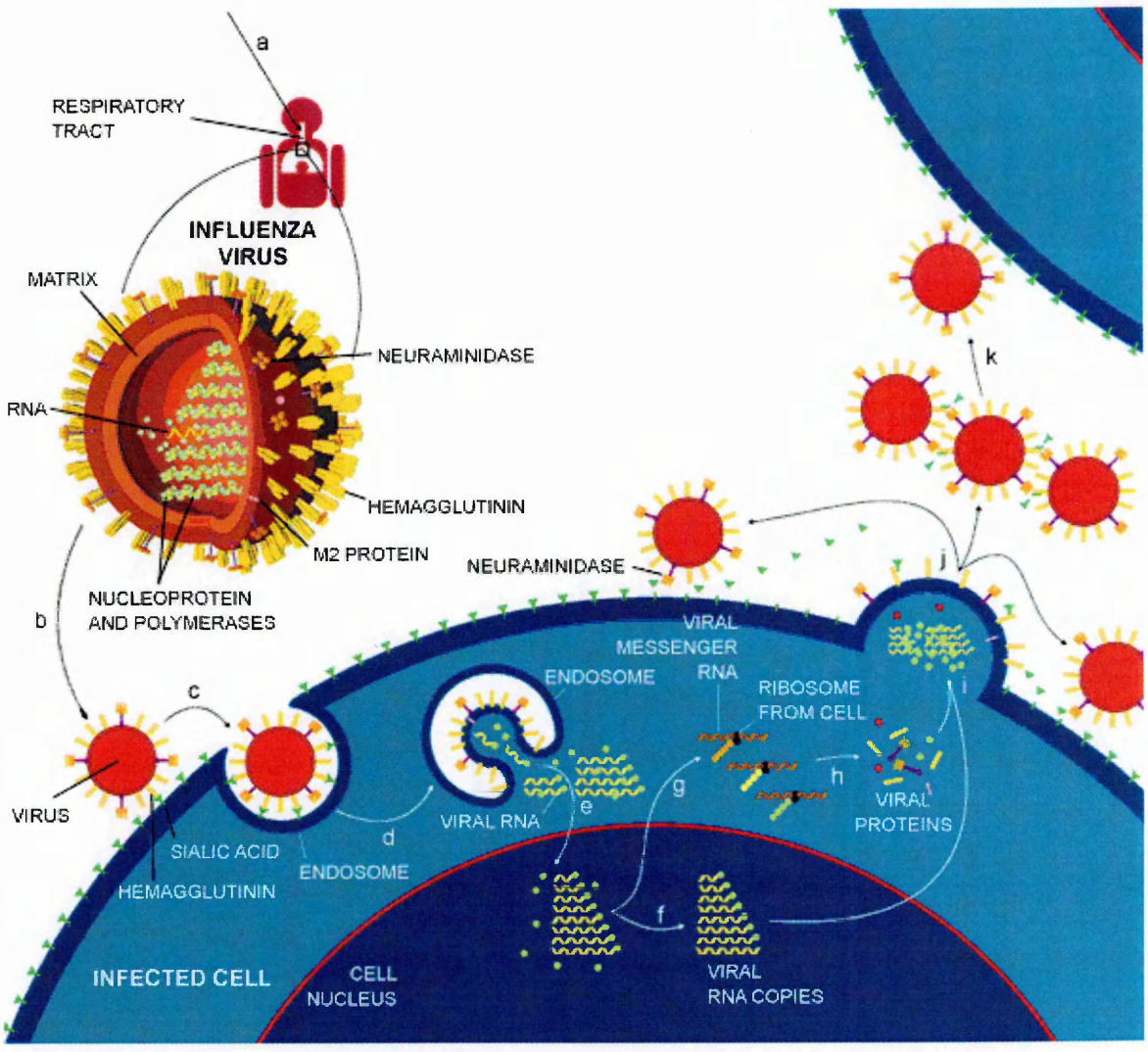


Figure 2 The virus life cycle (in humans) (Figure adapted from http://www.chemsoc.org/exemplarchem/entries/2001/sanderson/immunology.htm#work)

Influenza virus enters the respiratory tract **(a)** and attaches to cells by binding to sialic acid on cell surface receptors **(b)**. Virus enters the cell by endocytosis into endosomes **(c)**. Acidification of the endosome induces a conformational change in HA, leading to fusion of endosomal and viral membranes **(d)**. Genetic material is released into the cytoplasm and enters the nucleus of the cell **(e)**. In the nucleus, viral RNA is replicated **(f)** and transcribed into messenger RNA for the production of viral proteins in the cytoplasm **(g, h)**. Viral proteins and the viral genome are assembled at the cell surface and progeny virus is released by budding **(j)**. Viral neuraminidase removes sialic acid from the viral and cellular membranes and this virus can infect a new cell **(k)**.

1994). Prior to this event, the interior of the virion is acidified by action of the ion-channel M2 (Pinto et al., 1992; Bui et al., 1996; Chizhmakov et al., 1996). This leads to dissociation of M1 from the RNPs, a prerequisite for entry of the viral genetic material into the nucleus (Bukrinskaya et al., 1982; Martin and Helenius, 1991). Once in the nucleus, the vRNA is transcribed into messenger RNA (mRNA) and replicated by the polymerase complex proteins PA, PB1, PB2 (Hay et al., 1977; Braam et al., 1983; Honda et al., 2002). In addition to the polymerase proteins, NP is required for the synthesis of the vRNA (Beaton and Krug, 1986; Portela and Digard, 2002). The transmembrane proteins HA, NA and M2 are synthesised by endoplasmatic reticulum (ER)-associated ribosomes (Compans, 1973b; Hay, 1974; Lamb et al., 1985), and inserted into the membrane (McCauley et al., 1979; Bos et al., 1984; Hull et al., 1988). Folding, assembly into oligomeric structures and glycosylation occur in the ER, whereas processing of the glycans and palmitoylation take place in the Golgi (Schmidt, 1982; Gething et al., 1986; Sugrue et al., 1990; Gallagher et al., 1992; Saito et al., 1995). Upon assembly of the replicated viral genome and proteins at the apical side of the polarised cell (Boulan and Sabatini, 1978; Hughey et al., 1992; Kundu et al., 1996; Lin et al., 1998), the virus is released from the plasma membrane by budding mainly from lipid rafts (Skibbens et al., 1989; Scheiffele et al., 1999; Zhang et al., 2000). The NA facilitates this process by cleaving sialic acid from the cell surface, preventing aggregation of the virus at the cell surface (Palese et al., 1974b).

1.5 Immunity against Influenza

Protective immunity against influenza infection is primarily mediated by the B-cell response. Although antibodies are directed against any viral structural protein (HA, NA, M1, M2 and NP), neutralisation of virus is mediated by anti-HA antibodies (Virelizier et al., 1976). Serum immunoglobulin (Ig) G is mainly involved in this process (Virelizier,

1975; Palladino et al., 1995), although IgA and IgM may also contribute. Secretory IgA has been suggested to mediate mucosal immunity in the murine nasopharynx (Renegar and Small, 1991; Renegar et al., 2004) and has also been detected in nasal washes of influenza-immune humans (Clements et al., 1986). Serum antibodies are subtype-specific, and infection by one HA subtype confers little or no protective immunity to other subtypes (WHO, 1980). It has been shown that neutralisation occurs by blocking the interaction with receptors on host cells, thereby preventing internalisation of the virus (Hirst, 1942b; Taylor and Dimmock, 1985a; Taylor and Dimmock, 1985b; Knossow et al., 2002), although prevention of membrane fusion by some antibodies has also been suggested (Kida et al., 1983; Edwards and Dimmock, 2001).

$CD4^+$ T-lymphocytes (T-helper cells) are absolutely required for the production of antibodies against HA by signalling to HA-specific populations of B-cells to differentiate into antibody-secreting cells (Virelizier et al., 1974; Scherle and Gerhard, 1986). $CD8^+$ T-cells (cytotoxic T-cells) are largely cross-reactive (Zweerink et al., 1977) and mainly directed against the more conserved internal proteins PB1, PB2, PA and M1 (Bennink et al., 1987; Reay et al., 1989), with the major target being NP (Townsend et al., 1984; Yewdell et al., 1985). Although they are not protective and may not be absolutely required for the control and resolution of an influenza infection (Eichelberger et al., 1991; Scherle et al., 1992), they have been observed to enhance virus clearance from the lung (Mackenzie et al., 1989).

NA-specific immunity is also antibody-mediated but is unable to prevent infection (Webster and Laver, 1967; Kilbourne et al., 1968). However, subsequent virus replication might be suppressed by blocking release of virus from cells, leading to reduction of virus yield and spread (Schulman et al., 1968; Johansson et al., 1989).

Mice have proven to be a good model for the study of influenza immunity in man.

In the human population, however, the immune response is more complicated. In contrast to laboratory mice being naïve to individual influenza strains, the human population is subject to recurrent infection of antigenically distinct viruses. This results in the production of cross-reactive antibodies with reduced affinity (Francis et al., 1953; Fazekas de St. Groth and Webster, 1966). Therefore, concomitant immunity to previous infection might compromise the antibody repertoire to subsequent infection.

1.6 Influenza Surveillance and Vaccines

Recurrent influenza epidemics cause excess morbidity, particularly in children, and mortality in the elderly (Glezen, 1982), and result in an estimated 500,000 deaths per year worldwide (Stohr, 2002). Therefore, high-risk groups are protected against influenza infection by vaccination. Vaccines against influenza virus primarily stimulate humoral protective immunity. However, since the surface glycoproteins HA and NA evolve rapidly, giving rise to new infectious strains (see *1.7.5 Antigenic Variation, p.52*)„ the vaccine formula needs to be updated annually. The World Health Organisation (WHO) coordinates a global influenza surveillance network since 1952 (WHO; Payne, 1953), which routinely characterises the antigenic, genetic and epidemiological properties of currently circulating strains in order to include the prevalent viruses as vaccine components. These include type B and type A (H1N1 and H3N2) strains (trivalent vaccine). The viruses to be incorporated in the new vaccine are grown in eggs. Since many clinical isolates do not grow to high titres in this host cell system, HA and NA from the circulating H1N1 and H3N2 viruses are currently reassorted with a high yield donor (A/Puerto Rico/8/34) (Kilbourne, 1969; Kilbourne et al., 1971; Furminger, 1998). Although eggs are still the main substrate for vaccine production because of a general high yield of virus, this system presents drawbacks, e.g. the lack of reliable year-round supplies of high quality eggs, low

susceptibility of summer eggs to infection with influenza virus, the possible presence of pathogens and selection of antigenic variants (see *4.1.3 Host Cell-Mediated Variation, p.134*). Therefore, efforts have been made to improve tissue culture systems for large-scale virus propagation, with Madin-Darby canine kidney (MDCK) and green monkey kidney (VERO) cells being recommended as possible vaccine cell candidates (Bruhl et al., 2000; Halperin et al., 2002). Another new approach to vaccination is the use of reverse genetics (Neumann et al., 1999; Hoffmann et al., 2000) for possible production of influenza vaccines entirely from cloned viral complementary DNA (cDNA) (Subbarao et al., 2003; Ozaki et al., 2004).

Vaccines are produced from whole formalin-inactivated virus, from disrupted virus particles ("split" vaccines) and from purified surface antigens containing HA and NA ("subunit" vaccines). Since the "split" and "subunit" vaccines induce fewer side effects in the immunised people than the one prepared from whole virus, they represent the most widely used vaccines today. Inactivated vaccines are safe, immunogenic and effective, preventing illness in 70-90% of healthy young adults. However, such vaccines are considerably less effective ($\leq$ 50%) in the elderly, infants and patients with certain chronic diseases or immunodeficiency, and the duration of protection is relatively short (< 1 year) (Wright and Webster, 2001). Therefore, new strategies are being pursued to generate longer-lasting vaccines of improved immunogenicity (Kemble and Greenberg, 2003), e.g. by the use of adjuvants included in currently available vaccines (e.g. Babai et al., 2001; Podda, 2001; Rimmelzwaan et al., 2001) and the development of live attenuated influenza virus (LAIV) and DNA vaccines (reviewed by Wareing and Tannock, 2001; Ulmer, 2002). Of these, the LAIV vaccine has been extensively used in Russia and is nearing licensure in the United States.

1.7 Haemagglutinin

1.7.1 HA Synthesis and Transport

Haemagglutinin was originally named because of the ability of the virus to agglutinate erythrocytes (Hirst, 1941). It is encoded by RNA segment 4 and represents a type I integral membrane protein containing an (N)-terminal signal sequence (McCauley et al., 1979), a transmembrane domain near the carboxy (C)-terminus and a short cytoplasmic tail. HA is synthesised as a single polypeptide HA_0 (molecular weight (MW) ~ 75,000 daltons (Da)) in association with the rough ER (Compans, 1973b). Insertion into the membrane of the ER, cleavage of the signal peptide and core glycosylation all take place co-translationally (Compans, 1973a). Glycosylation of HA has been shown to be important for the correct folding and oligomerisation of HA into trimeric structures (MW ~ 220,000 Da) (Gallagher et al., 1992; Roberts et al., 1993; Hebert et al., 1995; Molinari and Helenius, 2000), which is essential for the transition of HA from the ER to the Golgi network (Gething et al., 1986; Copeland et al., 1988). Another post-translational modification involves the cleavage of precursor HA_0 into two disulphide-linked chains HA_1 (MW ~ 50,000 Da) and HA_2 (MW ~ 30,000 Da) (Lazarowitz et al., 1971; Klenk and Rott, 1973). As discussed below, proteolytic cleavage is a prerequisite for virus infectivity (Klenk et al., 1975; Lazarowitz and Choppin, 1975). Nonpathogenic viruses are cleaved extracellularly (Lazarowitz et al., 1971; Hay, 1974) by host-specific trypsin-like proteases (Lazarowitz et al., 1973a; Lazarowitz et al., 1973b; Klenk et al., 1977). The enzyme responsible for cleavage in the human respiratory tract has not been identified but may be similar to serine tryptase Clara, produced by cells of the rat bronchiolar epithelium, which has been shown to activate human influenza viruses (Kido et al., 1992). Some highly pathogenic H5 and H7 viruses are cleaved intracellularly (Klenk et al., 1974) by ubiquitous subtilisin-like proteases, such as furin (Stieneke-Grober et al., 1992). Accessibility to such proteases

appears to be due to the insertion of basic amino acids into the cleavage site or deletion of a carbohydrate in the vicinity, which, together with the wide tissue distribution of furin-like enzymes, is thought to be related to the high virulence and systemic infection of these viruses (for a review see Steinhauer, 1999).

1.7.2 The Structures of Haemagglutinin

Three different conformations adopted by HA have been defined structurally and are shown in Figure 3 and 4. The mature HA forms homotrimers of MW ~ 220,000 Da. As described above, each monomer is synthesised as a single polypeptide precursor HA_0, which is posttranslationally cleaved to generate HA with the C-terminus of HA_1 and the N-terminus of HA_2 being linked by a single disulphide bond between residue 14 of HA_1 and 137 of HA_2. This cleaved form of HA is present on the surface of infectious viruses (discussed below). Cleavage of HA is required for activation of the fusion potential and virus infectivity (Huang et al., 1980; White et al., 1981). The overall structures of cleaved and uncleaved HAs are superimposable, with only 19 residues being positioned differently (Wilson et al., 1981; Chen et al., 1998) (see Figure 3a and b). These form a prominent surface loop in HA_0 (shown in yellow) containing the cleavage site. Upon cleavage, the newly formed HA_2 N-terminus is buried in a negatively charged cavity at neutral pH. This N-terminal sequence contains a stretch of hydrophobic residues, which are highly conserved among influenza subtypes (Nobusawa et al., 1991). Both cleaved and uncleaved molecules project approximately 135 Å from the viral membrane and consist of two distinct regions, a globular and a stem domain. The globular domain at the membrane distal part of the molecule is composed entirely of HA_1 residues, which primarily form an eight-stranded anti-parallel β-sheet, corresponding to both the RBS (see *1.7.3.3 Haemagglutinin in Complex with Neu5Ac, p.36*) and the principal recognition sites for

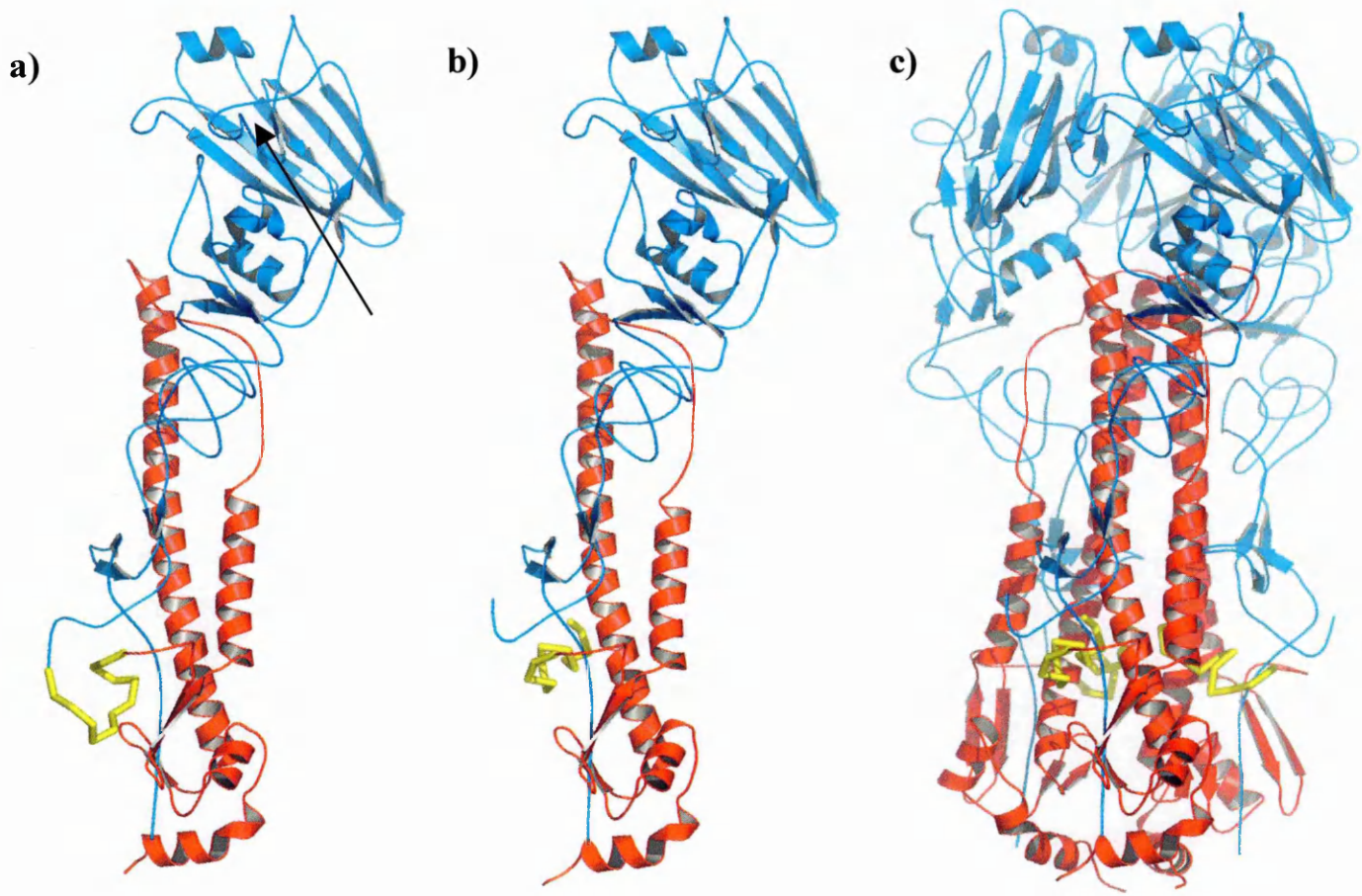


Figure 3 Ribbon representation of bromelain-released haemagglutinin (BHA) crystal structures
a) monomor of uncleaved precursor haemagglutinin (HA_0) **b)** monomer of cleaved haemagglutinin (HA) at neutral pH **c)** homotrimer of cleaved haemagglutinin. HA_1 residues are in blue, HA_2 residues are in red. The fusion peptide is shown in yellow. The arrow in **a)** points at the receptor-binding site.

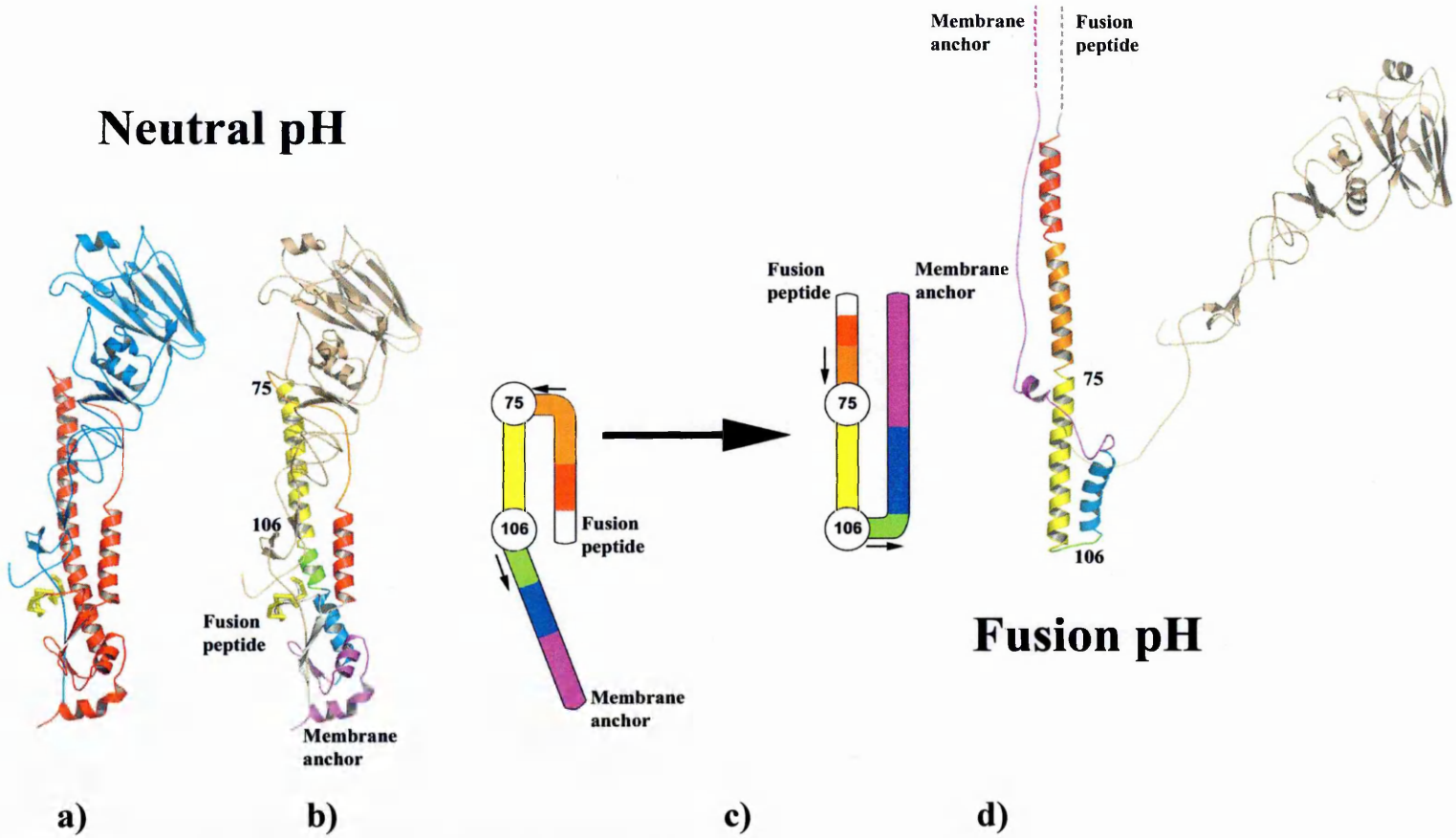


Figure 4 Ribbon representation of the structural changes that occur in HA at low pH

a) BHA monomer with stem domain (HA_2) in red, globular domain (HA_1) in blue and fusion peptide in yellow. **b)** BHA monomer with regions of HA_2 undergoing conformational changes at low pH indicated in different colours. **c)** Acidification of the endosome triggers drastic structural rearrangements of the HA_2 domain, which leads to the positioning of the fusion peptide and the membrane anchor region at the same end of the molecule **(d)**. The structure of the region comprising residues 75-106 is the same before and after the conformational change. The HA_1 domains retain their structures but de-trimerise.

neutralising antibodies (see *1.7.5.2.1 Antigenic Sites, p.54*). The stem domain extends 76 Å from the membrane and consists of residues of both HA_1 and HA_2. The main structural features of this domain are two α-helices connected by a hairpin loop. The HA trimer (Figure 3c) is stabilised by independent local contacts in the globular and stem regions. The HA_1 globular domains make pairwise contacts near the upper end of the molecule, whereas a triple-stranded coiled-coil of α-helices formed from the top half of the long helix from each monomer provides the major contacts in the HA_2 region (Wilson et al., 1981).

As described in *1.4 The Influenza Virus Life Cycle, p.22*, internalisation and subsequent acidification by the endosome triggers dramatic structural rearrangements in HA_2, leading to fusion of the viral and endosomal membranes. The low-pH conformation of HA is shown in Figure 4 and reveals the irreversible structural changes as a result of acidification (Bullough et al., 1994). The overall effect of these is the extrusion of the fusion peptide (the N-terminus of HA_2) from its buried position to the tip of the molecule. This conformational change leads to the positioning of the fusion peptide and the viral membrane anchor at the same end of HA, bringing the membranes into close proximity for fusion to occur.

Additionally, HA is co- and post-translationally modified. The molecule is highly N-glycosylated, the extent differing depending on the HA subtype, (Ha et al., 2002), and also contains three potential acylation sites at cysteine residues at the C-terminus of HA_2 (Kawaoka et al., 1990; Veit et al., 1991). The biological role of the oligosaccharides is described in *1.7.5.2.2 The Role of Oligosaccharides in Antigenic Drift, p.56*. All the structural information described above is derived from HA of A/Aichi/2/68, the prototype of the H3 subtype viruses responsible for the pandemic in 1968. Since then, the structure of the cleaved HA has been solved for several HAs of subtypes H1, H3, H5, H7 and H9 (Ha et al., 2002; Ha et al., 2003; Gamblin et al., 2004; Russell et al., 2004). These have

been shown to be structurally homologous.

1.7.3 Receptor-Binding

1.7.3.1 Discovery of Sialic Acid as Receptor Determinant for HA

As described above, influenza virus initiates infection by binding to cellular receptors, followed by endocytosis into endosomes. Earliest evidence for a specific cellular receptor was provided by Hirst, who demonstrated the agglutination of chicken red blood cells (RBC) by influenza virus at 4°C (Hirst, 1941; Hirst, 1942a). This was supported by the ability of the virus to adsorb to respiratory cells of ferret and mouse lungs (Hirst, 1943a; Fazekas de St. Groth, 1948a). Furthermore, interaction of influenza virus with a variety of mucopolysaccharides and mucoproteins, e.g. sputum mucoprotein (Marmion et al., 1953), was observed, leading to inhibition of haemagglutination (Burnet, 1951). However, these studies showed that a rise in incubation temperature to 37°C resulted in spontaneous and irreversible elution of virus from RBC and respiratory cells and also rendered the inhibitors inactive. The same effect was observed by incubation with an enzyme obtained from the culture filtrate of *Vibrio cholerae* (Burnet et al., 1946; Burnet, 1951). This enzyme was therefore called receptor-destroying enzyme (RDE) (Burnet and Stone, 1947). The experiment that pre-treatment of the intact mouse lung with RDE prevented infection by influenza virus left little doubt on the essential role of cellular receptors for initiation of virus infection (Stone, 1948). The similarity of effects exhibited by virus and RDE suggested the existence of an enzyme on the virus envelope, and first chemical evidence for its activity was provided by incubation of ovomucin with influenza virus (Gottschalk and Lind, 1949). A product with a low molecular weight was released, which was later identified as N-acetylneuraminic acid (see below) (Klenk and Lempfrid, 1955). Further evidence for sialic acid as the cellular receptor determinant was provided by later studies

showing that binding of virus to RDE (now called neuraminidase)-treated cells could be restored by enzymatic reconstitution with sialic acid and by incubation with exogenous sialogangliosides (Paulson et al., 1979; Bergelson et al., 1982; Suzuki et al., 1985).

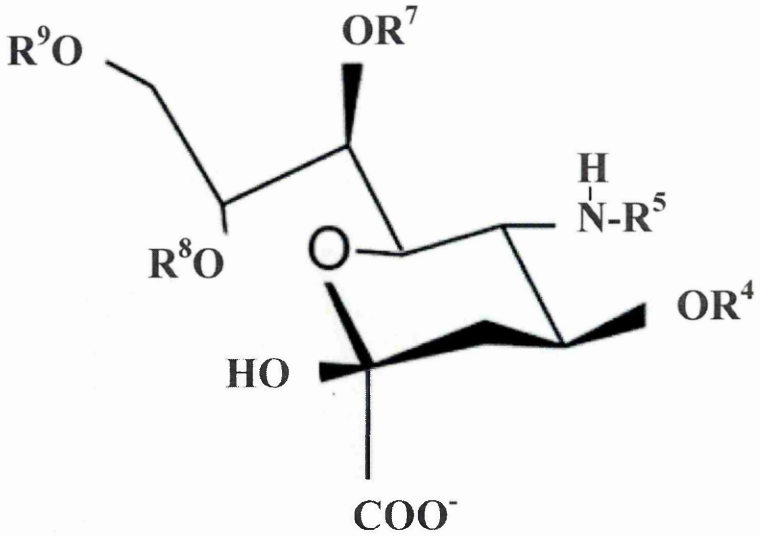
Although HA interacts with both sialic acid-containing glycoproteins and glycolipids in receptor-binding studies, the results of a recent studies suggest that endocytosis into host cells specifically requires N-linked sialoglycoproteins (Chu and Whittaker, 2004).

1.7.3.2 The Structure and Functions of Sialic Acids

Sialic acids represent a family of over forty 9-carbon carboxylated sugars, which are found in higher invertebrates, vertebrates and some micro-organisms (reviewed by Schauer, 1982) (see Figure 5a). The most common is N-acetylneuraminic acid (2-keto-5-acetamido-3,5-dideoxy-D-glycero-D-galacto-nonulopyranos-1-onic acid) (Neu5Ac), which is thought to be the biosynthetic precursor for all other members (see Figure 5b). Hydroxylation of the N-acetyl group gives N-glycolylneuraminic acid (Neu5Gc). Further structural diversity is generated by various substitutions at the 4, 5, 7, 8 and 9-carbon positions, most commonly by O-acetylation at positions 4, 7, 8 and 9.

Sialic acids are components of a variety of oligosaccharides, polysaccharides, glycoproteins and glycolipids on cell membranes and secreted molecules and are usually found at the terminal positions. The linkage involves α-glycosidic bonds between the C-2 anomeric hydroxyl group of sialic acid and most commonly the C-3 or C-6 hydroxyl group of the penultimate galactose (Gal), N-acetylgalactosamine (GalNAc) or N-acetylglucosamine (GlcNAc) (α(2,3)- and α(2,6)-linkage, respectively). Where sialic acids are linked to each other, they usually form α(2,8)-bonds. Sialic acids have been implicated in various biological processes. By virtue of their negative charge they can mask

a)



b)

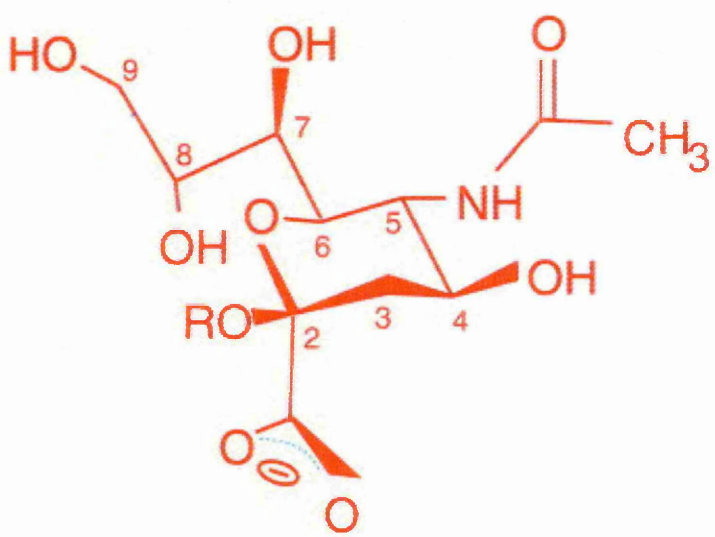


Figure 5 Structures of sialic acids
a) Core structure of naturally occurring sialic acids Structural diversity is given by differences of the chemical composition of the side-groups (R).
R^4 = H, acetyl
R^5 = acetyl, glycolyl
R^7 = H, acetyl
R^8 = H, acetyl, methyl, sulphate
R^9 = H, acetyl, L-lactyl, phosphate

b) Struture of neuraminic acid (Neu5Ac) R is the next monosaccharide

intermolecular and intercellular interactions, such as immune and receptor recognition, and hinder the action of proteases and endoglycosidases (Schauer et al., 1984; Schauer, 1985). However, sialic acids also act as ligands in recognition phenomena involving lectins, such as selectins, I-type lectins, bacterial and viral lectins, and thereby play a regulatory role in vascular, immunological, neuronal and infection processes (Varki, 1997).

Many studies have focused on the roles of O- and N-substituted sialic acids in biological processes (for a review see Schauer, 1991). O-acetylation at position 4, 7, and 9, renders complex carbohydrates more resistant to sialidase action and thus extends the lifetime of e.g. serum glycoproteins and RBC. Notably, 9-O-acetylation inhibits binding of influenza A viruses (Higa et al., 1985) while mediating binding of influenza C viruses (Herrler et al., 1985).

1.7.3.3 Haemagglutinin in Complex with Neu5Ac

When the structure of A/Aichi/2/68 HA was first determined, a shallow depression at the membrane-distal tip of the molecule was found to be lined with amino acids which had been conserved since the emergence of the H3 subtype in humans in 1968 (Wilson et al., 1981). It was therefore suggested that this pocket might form the binding site for the receptor, which was consequently confirmed by X-ray crystallography of HA in complex with Neu5Ac (Weis et al., 1988). As seen in Figure 6, the axial carboxylate, the acetamido-nitrogen and the 8- and 9-hydroxyl groups of the glycerol side-chain of Neu5Ac orient into the pocket, whereas the 4-hydroxyl points out of the site into solution. The pocket is defined by three secondary structural elements. The 190-helix (residues 190 to 198) forms the "back" of the site, whereas the "sides" are composed of the 130-loop (residues 135-138) and the 220-loop (residues 221-228). Conserved residues Tyr98, His183, Tyr195 and Trp153 form the "base" of the site (see Appendix 1 for amino acid

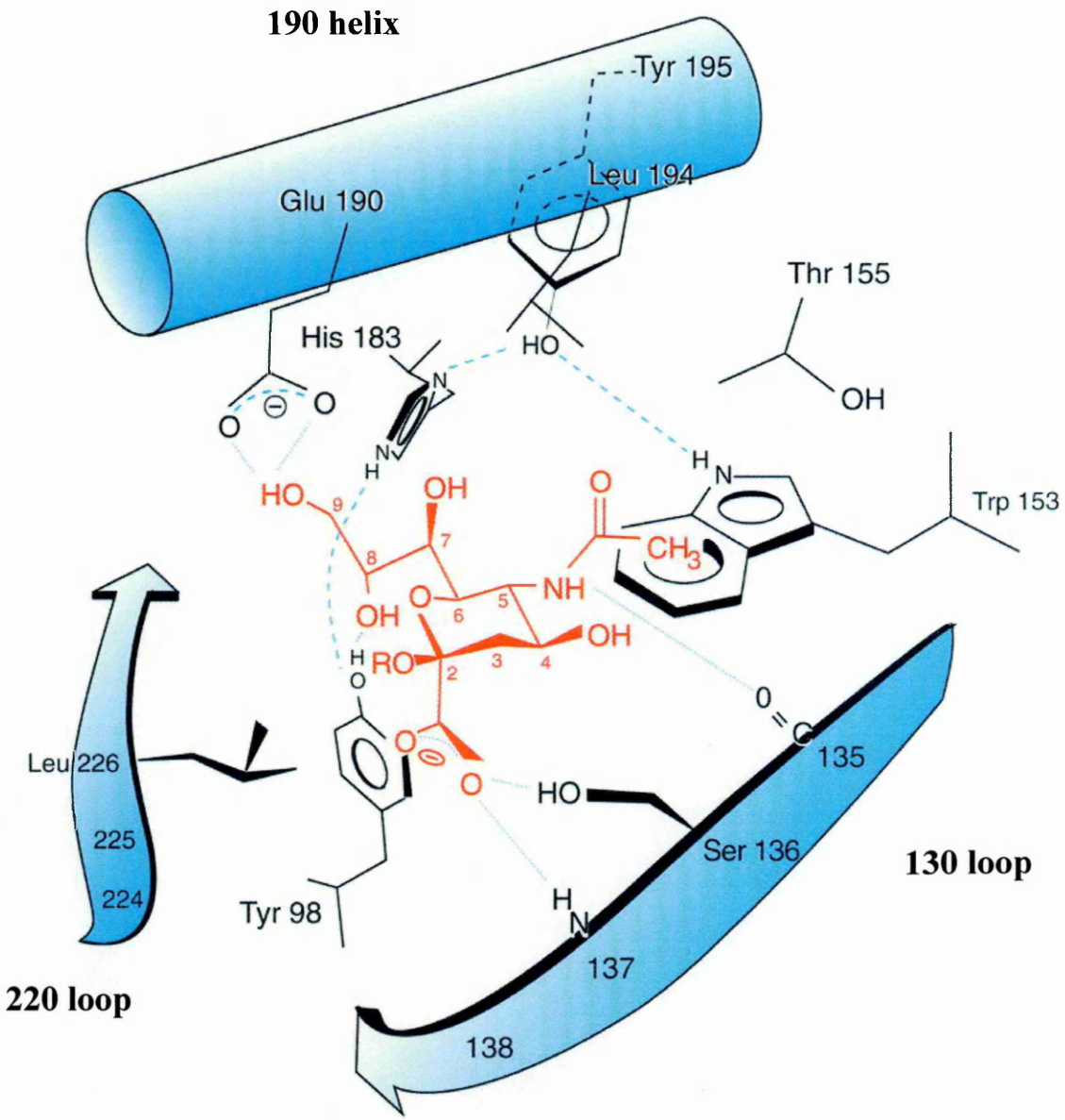


Figure 6 Schematic diagram of the HA receptor-binding site complexed with neuraminic acid (coloured in red) The three main secondary structure elements of the receptor-binding pocket are indicated in blue. Conserved residues Tyr98, His153, Tyr195 and Trp153 form the "base" of the site. Potential hydrogen bonds are indicated as dotted blue lines.

three- and one-letter codes, p.254). A number of potential hydrogen bonds are formed between conserved residues and between several of these residues and Neu5Ac (shown in blue in Figure 6). Additional conserved residues behind the pocket (Cys97, Pro99, Cys139, Phe147, Tyr195, Arg229) may be important in stabilising the architecture of the RBS (Wiley and Skehel, 1987).

A number of studies have assessed the importance of the functional groups of Neu5Ac in the interaction with HA and identified the N-acetamido group, the orientation of the carboxygroup and the glycerol chain as critical determinants (Sauter et al., 1989; Hanson et al., 1992; Kelm et al., 1992; Sauter et al., 1992). The contributions of residues in the RBS to the interaction with Neu5Ac has been assessed by mutagenesis studies (Martin et al., 1998) and highlighted the importance of the hydrogen network linking residues Tyr98, His183, Tyr195 and Trp153. The structure of HA in complex with Neu5Ac has since been solved for HA of different subtypes, with the receptor determinant being bound essentially in the same way (Ha et al., 2001; Gamblin et al., 2004).

1.7.4 Receptor Specificity

1.7.4.1 Host-Range Restriction

Influenza A virus infects a variety of hosts, including humans, pigs, horses, sea mammals, poultry, wild ducks, and other migrating waterfowl. All 16 HA subtypes are perpetuated in aquatic birds, which appear to be the reservoir of all influenza viruses for other species (for a review see Webster et al., 1992). Of these, 3 HA subtypes have evolved in humans (H1, H2, H3), at least 2 in pigs (H1, H3) and 2 in horses (H3, H7). Despite the common origin of influenza viruses, their host range appears to be restricted. Experimental infections have shown poor replication of avian viruses in primates (Murphy et al., 1982; Snyder et al., 1987; Beare and Webster, 1991), while human isolates do not replicate efficiently in ducks

(Webster et al., 1978; Kida et al., 1980; Hinshaw et al., 1983). However, restriction is considered to be partial due to frequent infection of generally unsusceptible hosts. Such interspecies transmission has been observed from birds to humans (Subbarao et al., 1998; Peiris et al., 1999; Fouchier et al., 2004), pigs (Brown et al., 1994; Karasin et al., 2000; Peiris et al., 2001) and sea mammals (Geraci et al., 1982; Hinshaw et al., 1984; Hinshaw et al., 1986), from pigs to humans (Claas et al., 1994; Gregory et al., 2001) and humans to pigs (Peiris et al., 2001; Marozin et al., 2002). However, these viruses are not usually maintained in the infected species, probably due to inefficient replication as a result of poor genetic fit to these hosts.

1.7.4.2 Correlation between Receptor-Linkage Specificity and Host of Origin

Host range restriction appears to be a polygenetic trait and the contribution of each gene product has not been fully elucidated (Scholtissek et al., 1985; Snyder et al., 1985; Tian et al., 1985; Snyder et al., 1987; Subbarao et al., 1993). However, HA has been the focus as a primary determinant due to its role in host cell recognition. Soon after sialic acid was discovered as the essential component of receptors, it became clear that different influenza strains varied in their recognition of certain receptor analogues (e.g. Burnet, 1948; Choppin and Tamm, 1960). In particular, the ability of certain viruses to distinguish between the α(2,6)- and α(2,3)-linkage of sialic acid to Gal was detected (Paulson et al., 1979; Carroll et al., 1981). Importantly, a correlation between receptor-binding specificity and host species, from which the virus was isolated, was reported. Whereas human viruses preferentially recognised α(2,6)-linked sialic acid, isolates from birds and horses showed preference for the α(2,3)-linkage (Rogers and Paulson, 1983; Rogers and D'Souza, 1989; Connor et al., 1994). This observation was confirmed in subsequent studies (Matrosovich et al., 1993; Gambaryan et al., 1997; Matrosovich et al., 1997; Ito et al., 1998). In contrast,

porcine viruses are more heterogeneous in their recognition phenotype. The studies mentioned above showed that some pig isolates display a clear avian-like, others a more human-like receptor recognition, whereas a third group is distinguished by relatively small differences in its affinity for the two linkages. In addition, a correlation between amino acids at positions 226 and 228 of the H2 and H3 subtype and receptor specificity was observed (numbering based on H3 HA, Winter et al. (1981)). Human isolates that preferentially bound the α(2,6)-linkage contained Leu226 and Ser228, whereas avian viruses recognising the α(2,3)-linkage contained Gln226 and Gly228 (Connor et al., 1994).

The main evidence for the ability of residue 226 to modulate receptor specificity has been provided by growth of an H3 human virus in the presence of horse serum, which has been shown to be an inhibitor of both H2 and H3 subtype viruses of human origin (Cohen and Belyavin, 1959; Rogers et al., 1983b). The inhibitory effect of horse serum has been attributed to α_2-macroglobulin, a glycoprotein containing mainly α(2,6)-linked sialic acid (Hanaoka et al., 1989). A resistant mutant was selected that changed its linkage preference from α(2,6) to α(2,3) and contained the single amino acid change Leu226Gln (Rogers et al., 1983a; Rogers et al., 1983b). H2 variants isolated by the same method (Choppin and Tamm, 1959; Choppin and Tamm, 1960) revealed the same changes in specificity and sequence, with an additional change Ser228Gly (Connor et al., 1994). Selection in the reverse direction has also been accomplished with an avian H3 virus that acquired α(2,6)-linkage recognition and a Leu226, typical for human isolates (Rogers et al., 1985). However, other residues in addition to 226 and 228 have been shown to modulate receptor specificity, e.g. 218, 193 and 205 (Daniels et al., 1987; Suzuki et al., 1989) (see *1.7.6 Interrelationship between Antigenic Variation and Receptor-Binding Properties, p.58*).

In contrast to the H2 and H3 subtypes, amino acids 226 and 228 appear not to be

directly implicated in receptor specificity for the H1 subtype viruses, since they generally contain Gln226 and Gly228, irrespective of their linkage preference. Other amino acids have been proposed to be involved in determining specificity, namely 138, 186, 190, 194 and 225 (Rogers and D'Souza, 1989; Matrosovich et al., 1997; Gambaryan et al., 1999; Mochalova et al., 2003). As for the H2 HA, the H1 HA amino acid numbering is based on the H3 subtype (Winter et al., 1981).

A recent study has shown the ability of an influenza virus to recognise sialic acid in the α(2,8)-linkage, in addition to the α(2,3)- and α(2,6)-linkages described above (Wu and Air, 2004). The biological significance of the interaction with α(2,8)-linked sialic acid and possible differences in its recognition for viruses of different subtypes and host origin remains to be shown.

1.7.4.3 Molecular Mechanism for Receptor Specificity

1.7.4.3.1 H3, H5 and H9 Subtype

The molecular mechanism involved in linkage recognition has been addressed with X-ray studies of HA in complex with receptor analogues containing Neu5Ac either in the α(2,3)- or the α(2,6)-linkage (Eisen et al., 1997; Ha et al., 2001; Ha et al., 2003). The role of amino acids at position 226 and 228 has been assessed by these receptor analogues bound to a human H3 (Leu226, Ser228), an avian H3 (Gln226, Gly228), an avian H5 (Gln226, Gly228) and a swine H9 subtype (Leu226, Gly228). As can be seen in Figures 7-10, the orientation of Neu5Ac in the site is essentially identical. However, the preferred receptor analogues are bound in different conformations. The α(2,3)-linkage is bound in a *trans* and the α(2,6)-linkage in a *cis* conformation around the glycosidic bond of Neu5Ac to the penultimate Gal, respectively. These enable different atomic contacts between the glycosidic linkage and residues in the binding site. Whereas the α(2,6)-motif appears to be

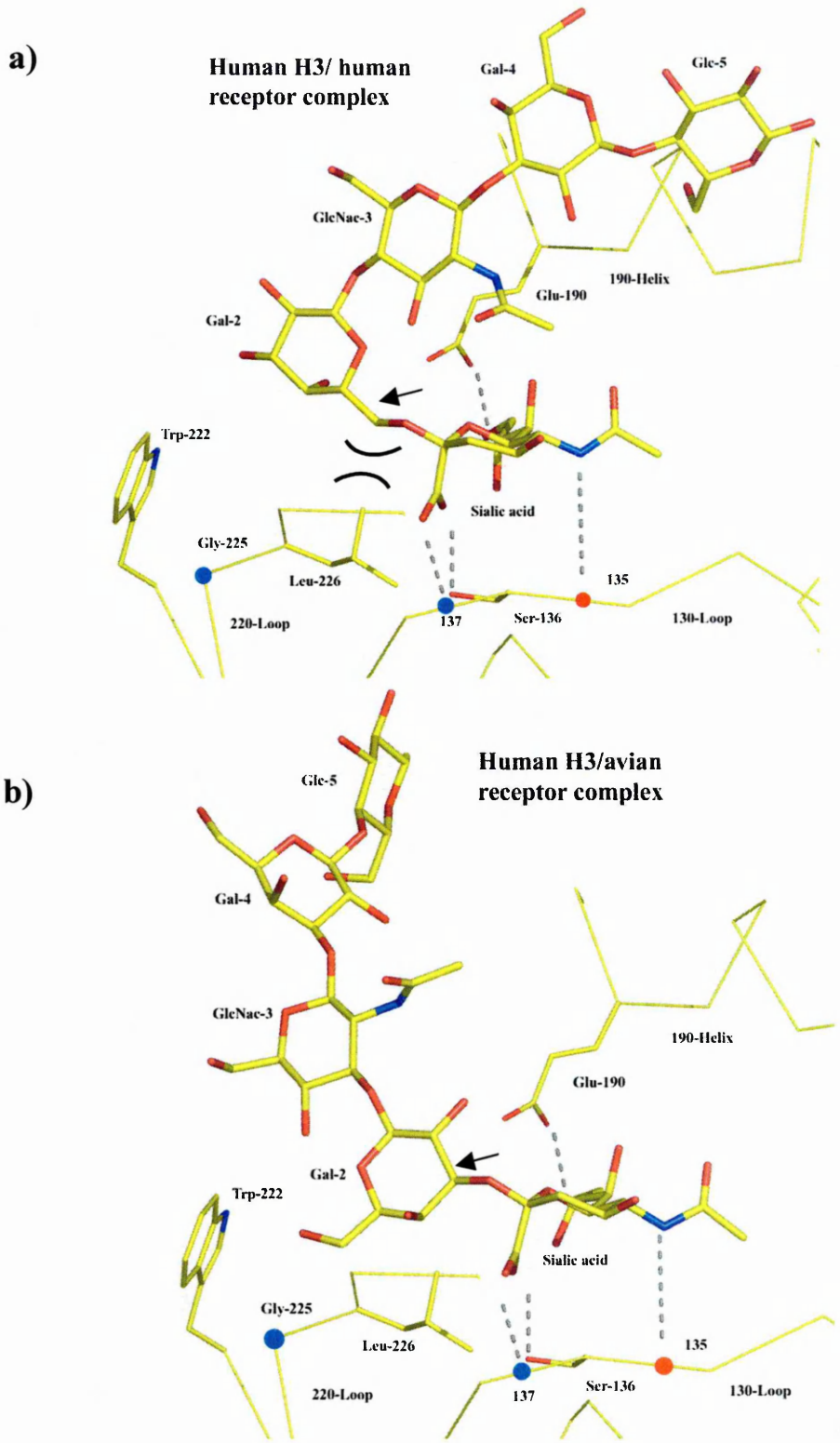


Figure 7 Structures of human H3 HAs (X31) complexed with receptor analogues
Potential hydrogen bonds are shown in grey broken lines. Residues making interactions via main-chain nitrogens are shown as blue spheres and those interacting via main-chain carbonyl-groups as red spheres. The receptor analogues are coloured in yellow for carbon, red for oxygen and blue for nitrogen atoms **a)** The α(2,6)-linked receptor analogue (LSTc) is bound in *cis* (indicated by the almost horizontal arrow) enabling the non-polar Leu226 to make van der Waals contacts with Gal-2, as indicated by semicircles **b)** The α(2,3)-linked receptor analogue (LSTa) is also bound in *cis*.

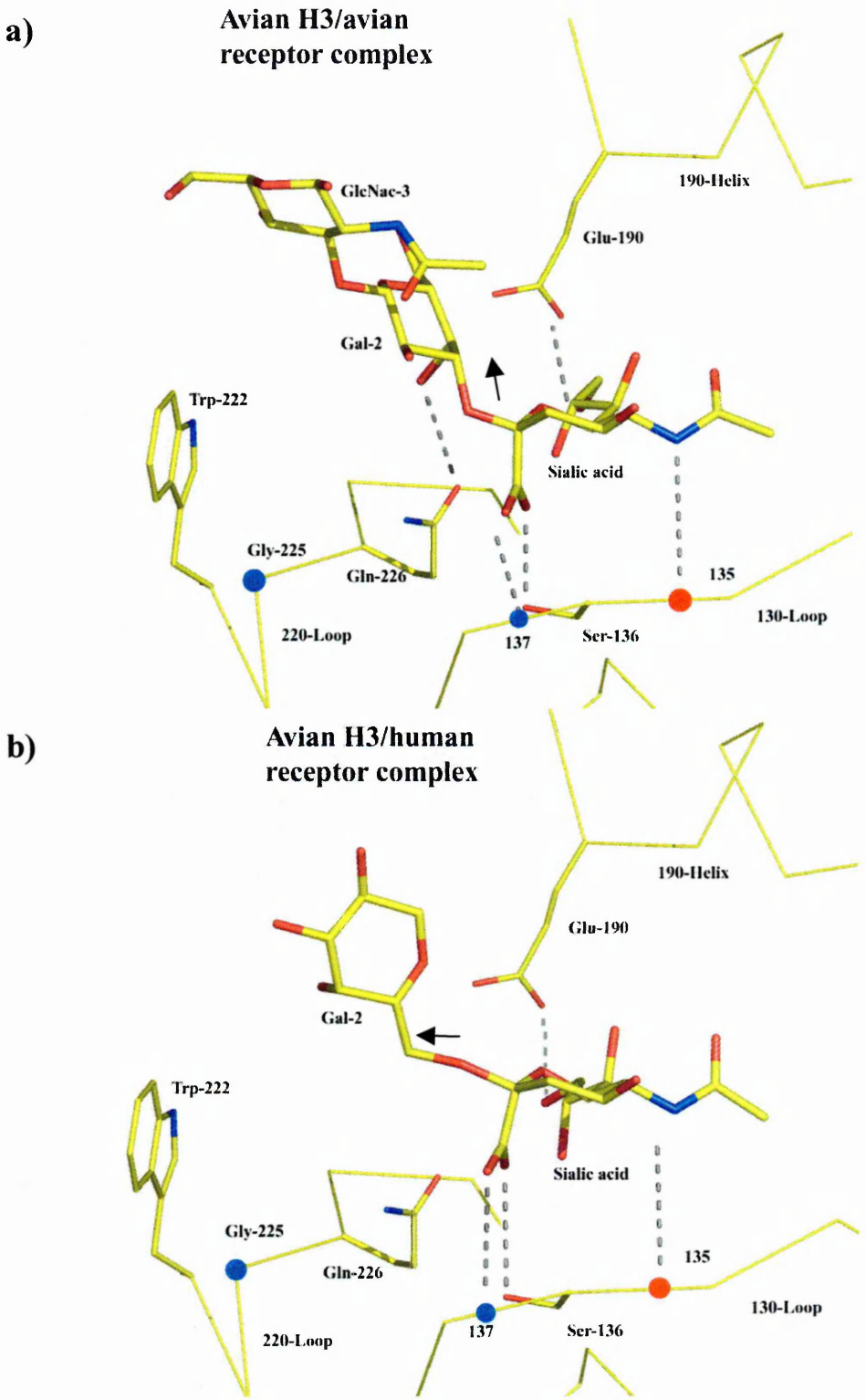


Figure 8 Structures of avian H3 HAs (Dk/Ukraine/1/63) complexed with receptor analogues Potential hydrogen bonds are shown in grey broken lines. Residues making interactions via main-chain nitrogens are shown as blue spheres and those interacting via main-chain carbonyl-groups as red spheres. The receptor analogues are coloured in yellow for carbon, red for oxygen and blue for nitrogen atoms **a)** The α(2,3)-linked receptor analogue (LSTa) is bound in *trans* (indicated by the almost vertical arrow) enabling the polar Gln226 to form a hydrogen bond to Gal-2 **b)** The α(2,6)-linked receptor analogue (LSTc) is bound in *cis* (indicated by the almost horizontal arrow). Only two saccharides are ordered.

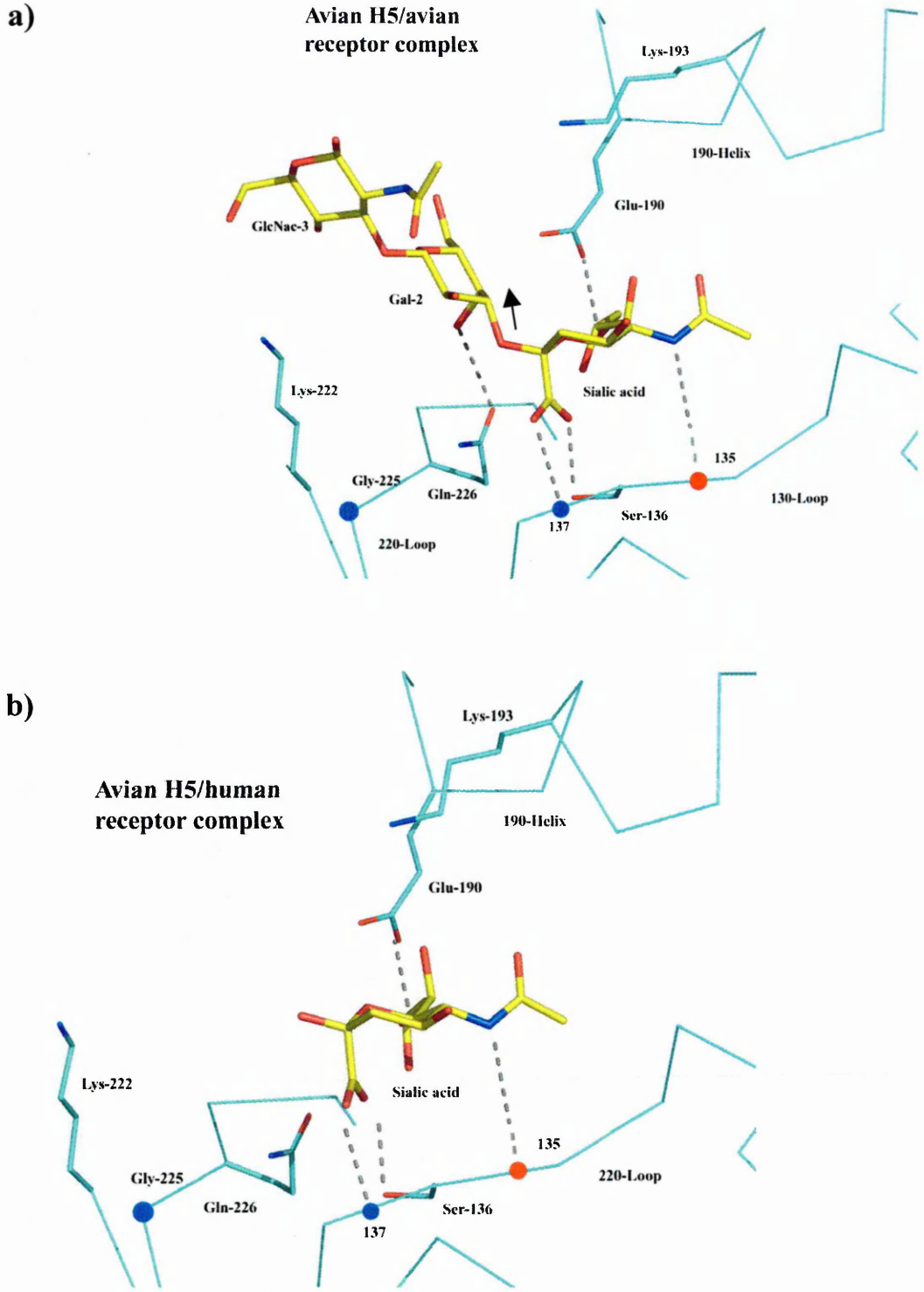


Figure 9 Structures of avian H5 HAs (Dk/Singapore/3/97) complexed with receptor analogues Potential hydrogen bonds are shown in grey broken lines. Residues making interactions via main-chain nitrogens are shown as blue spheres and those interacting via main-chain carbonyl-groups as red spheres. The receptor analogues are coloured in yellow for carbon, red for oxygen and blue for nitrogen atoms **a)** The α(2,3)-linked receptor analogue (LSTa) is bound in *trans* (indicated by the almost vertical arrow) enabling the polar Gln226 to form a hydrogen bond to Gal-2 **b)** Only sialic acid is visible for the bound α(2,6)-linked receptor analogue (LSTc).

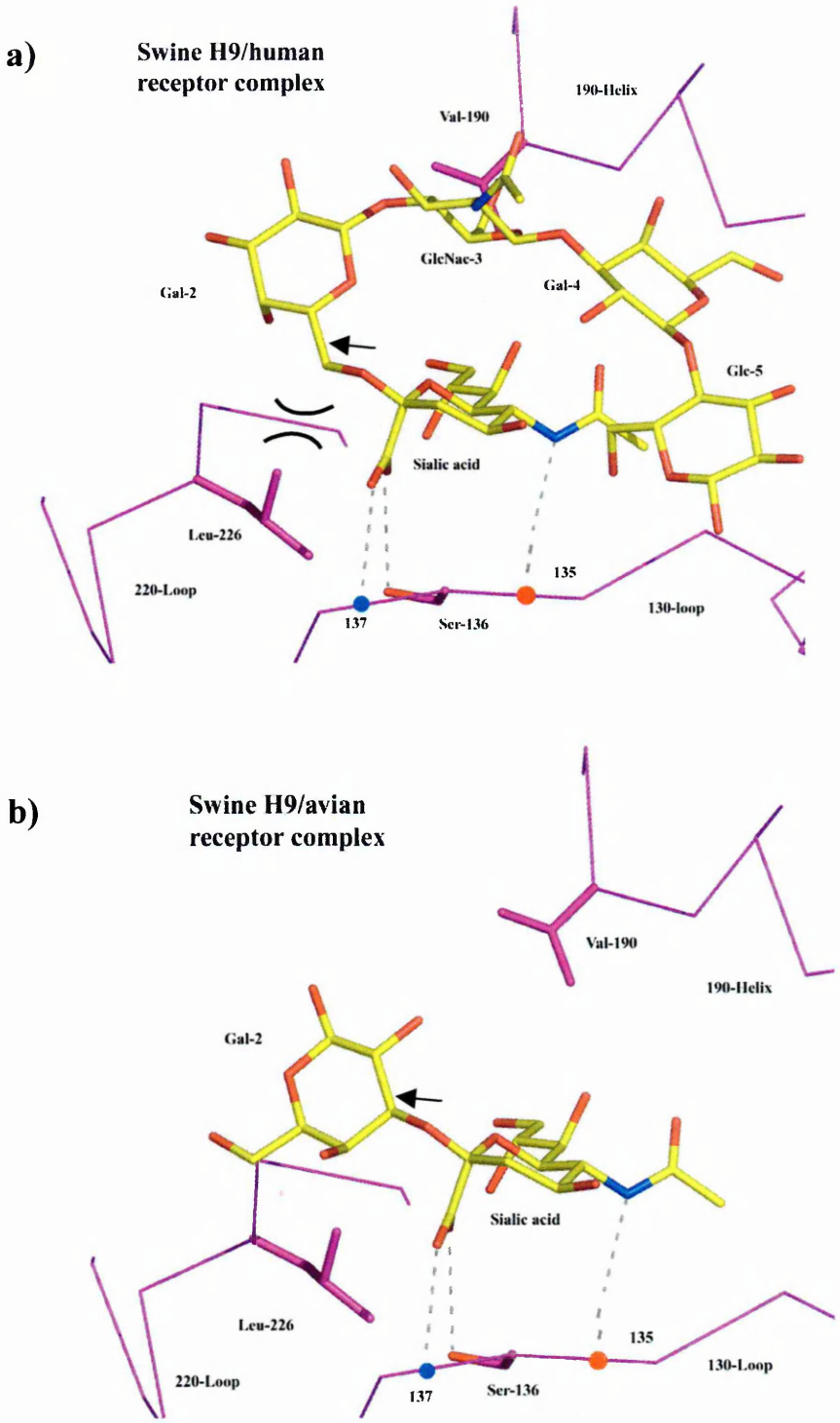


Figure 10 Structures of swine H9 HAs (Sw/Hong Kong/9/98) complexed with receptor analogues Potential hydrogen bonds are shown in grey broken lines. Residues making interactions via main-chain nitrogens are shown as blue spheres and those interacting via main-chain carbonyl-groups as red spheres. The receptor analogues are coloured in yellow for carbon, red for oxygen and blue for nitrogen atoms **a)** The α(2,6)-linked receptor analogue (LSTc) is bound in *cis* (indicated by the almost horizontal arrow) enabling the non-polar Leu226 to make van der Waals contacts with Gal-2 (indicated by semicircles) **b)** The α(2,3)-linked receptor analogue (LSTa) is also bound in *cis*. Only two saccharides are ordered.

stabilised by van der Waals interactions involving Leu226, Gln226 mediates hydrogen bonds with the α(2,3)-motif. In contrast, the non-preferred combinations of Leu226 in the α(2,3)- and Gln226 in the α(2,6)-complex result in unfavourable interactions of these residues with the glycosidic linkage. Comparison of the HA structures also revealed that the width of the avian binding site is narrower than the human site, probably dictated by the amino acid combinations at positions 226 and 228. The binding site of a swine H9 containing a human-specific Leu226 and an avian-specific Gly228 has been shown to be of intermediate size. The size of the binding site may therefore optimise the interaction with the respective linkage, with the Leu226/Gly228 representing an evolutionary intermediate between an avian and a human RBS.

1.7.4.3.2 H1 Subtype

HA crystal structures of the H1 subtype human 1918 pandemic virus and the earliest human (A/Puerto Rico/8/34) and swine (A/Sw/Iowa/15/30) viruses have recently become available (Gamblin et al., 2004; Stevens et al., 2004). The structures of the human-1934 and swine HA bound to receptor analogues have also been solved and the residues involved in receptor-recognition identified, as shown in Figures 11 and 12 (Gamblin et al., 2004). The human-1934 HA is found in complex with both the α(2,3)- and α(2,6)-linked receptor analogues, in agreement with earlier studies showing "dual" receptor specificity for early H1 isolates (Rogers and Paulson, 1983; Rogers and D'Souza, 1989). In the swine HA-α(2,6)-complex, all five sugars are detected, whereas only Neu5Ac is visible for the α(2,3)-linked receptor analogue. The main interactions involve hydrogen bonds between residues 222, 225 and Gal-2. This is a result of a lower positioning of Gal into the site as compared to the H3/H5/H9 subtypes and appears to be due to slightly different conformations of the 130 and 220 loops. In the swine HA, additional contacts of Asp190 to

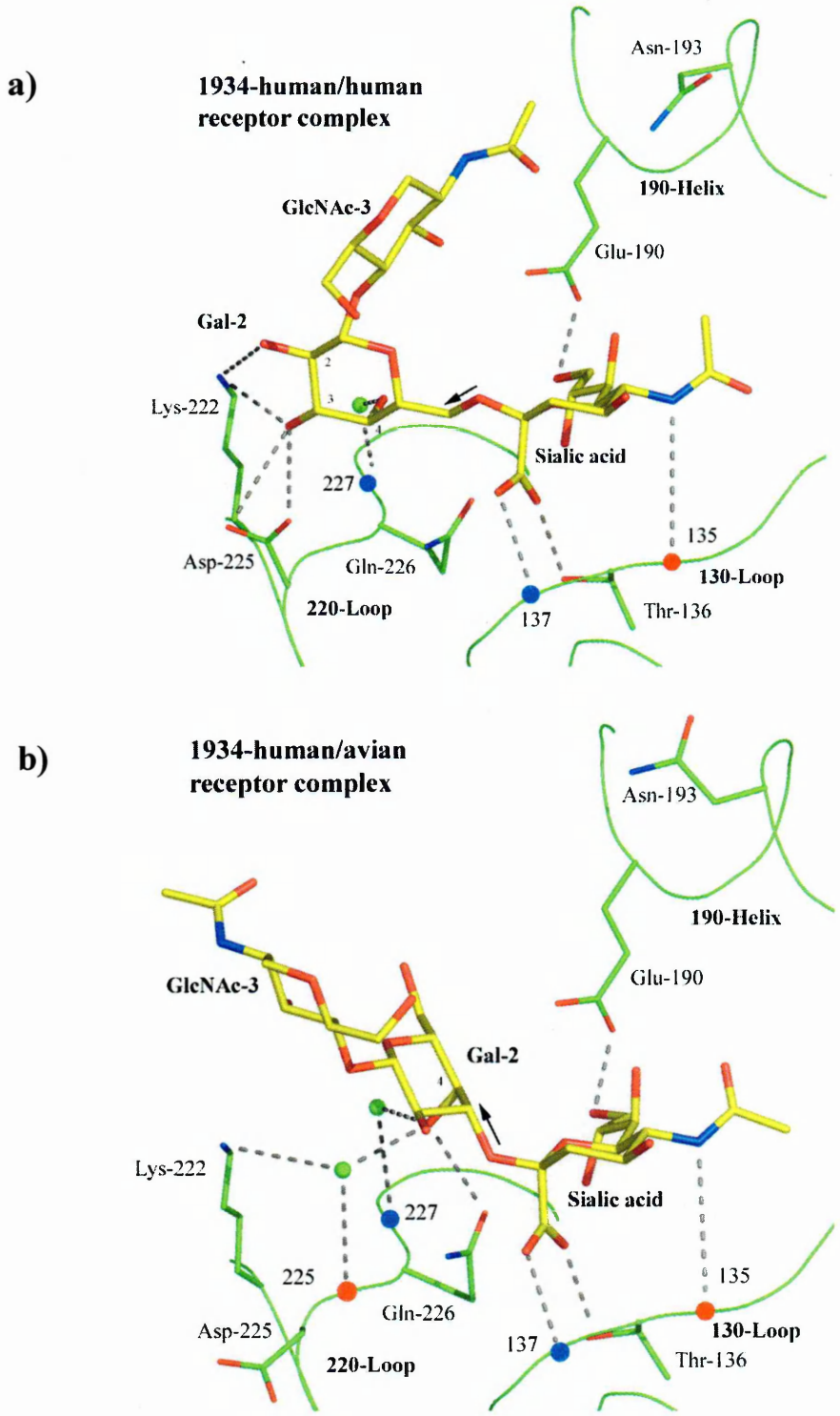


Figure 11 Structures of human H1 HAs (Puerto Rico/8/34) complexed with receptor analogues Potential hydrogen bonds are shown in grey broken lines. Residues making interactions via main-chain nitrogens are shown as blue spheres and those interacting via main-chain carbonyl-groups as red spheres. Water molecules are indicated by green spheres. The receptor analogues are coloured in yellow for carbon, red for oxygen and blue for nitrogen atoms **a)** The α(2,6)-linked receptor analogue (LSTc) is bound in *cis* (indicated by the almost horizontal arrow) **b)** The α(2,3)-linked receptor analogue (LSTa) is bound in *trans*, (indicated by the almost vertical arrow).

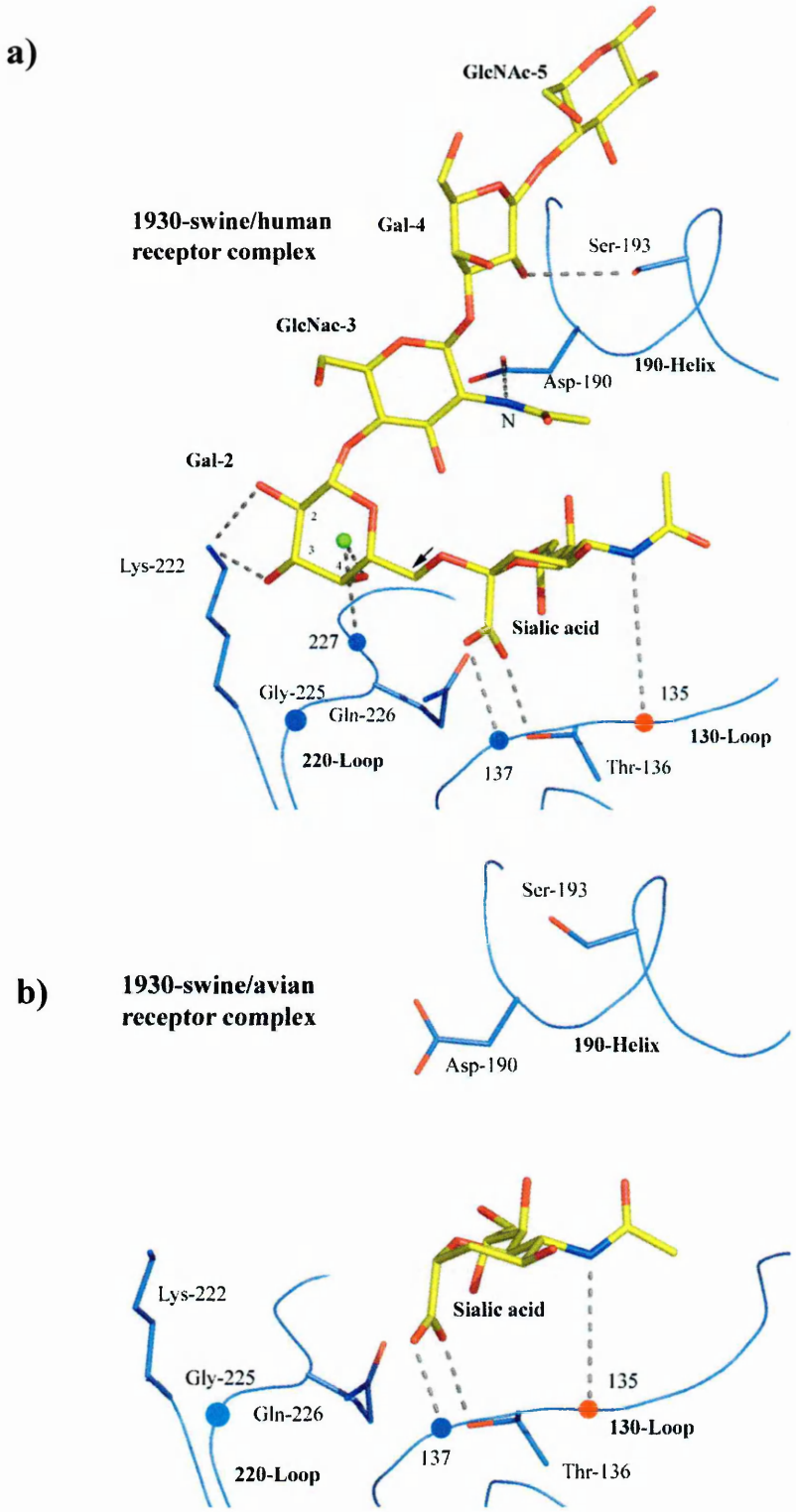


Figure 12 Structures of swine H1 HAs (Sw/Iowa/15/30) complexed with receptor analogues Potential hydrogen bonds are shown in grey broken lines. Residues making interactions via main-chain nitrogens are shown as blue spheres and those interacting via main-chain carbonyl-groups as red spheres. Water molecules are indicated by green spheres. The receptor analogues are coloured in yellow for carbon, red for oxygen and blue for nitrogen atoms a) The α(2,6)-linked receptor analogue (LSTc) is bound in *cis* (indicated by the almost horizontal arrow) b) Only sialic acid is visible for the bound α(2,3)-linked receptor analogue (LSTa).

the N-acetyl of GlcNAc and the Ser193 to Gal-4 are observed in the α(2,6)-linked complex. This finding would explain the preferential binding of 6'-sialyllactosamine (6'SLN, Neu5Acα(2,6)Galβ(1,4)GlcNAc) over 6'sialyllactose (6'SL, Neu5Acα(2,6)Galβ(1,4)Glc) by human H1 viruses (Matrosovich et al., 1993), which generally contain Asp190 (Matrosovich et al., 1997). In contrast to the H3/H5/H9 subtypes, Gln226 plays a passive role in recognition of the α(2,6)-linkage by being positioned lower in the binding site, thereby preventing unfavourable interactions. However, Gln226 appears to be involved in stabilising the α(2,3)- complex by formation of hydrogen bonds with Gal-2. The requirement of Glu190 in this process has been suggested and the presence of Asp190 might explain the unfavourable interaction of the swine HA with this linkage.

In addition, the structures of the H1 and H3 subtype HAs complexed with receptor analogues reveal that, whereas the α(2,3)-linkage is always bound in a similar way, the conformation of the α(2,6)-linked receptor analogue varies (Figure 13) (Gamblin et al., 2004).

1.7.4.4 Host Cell Factors Involved in Selection of Linkage Specificity

Evolution of receptor specificity may be the result of selective pressure exerted by the linkage of sialic acids on host cells. This theory is based on studies with linkage-specific lectins showing a correlation of host tropism of influenza viruses with the predominance of a specific linkage in host tissues. In humans, influenza viruses replicate in the ciliated epithelium of conducting airways (Hers, 1966), although neither initial targets of viral infection nor specific cell types essential for viral replication have been defined. Initially, it was shown that human tracheal epithelia cells are abundant in α(2,6)-linked sialic acid (Baum and Paulson, 1990; Couceiro et al., 1993). However, recent studies have

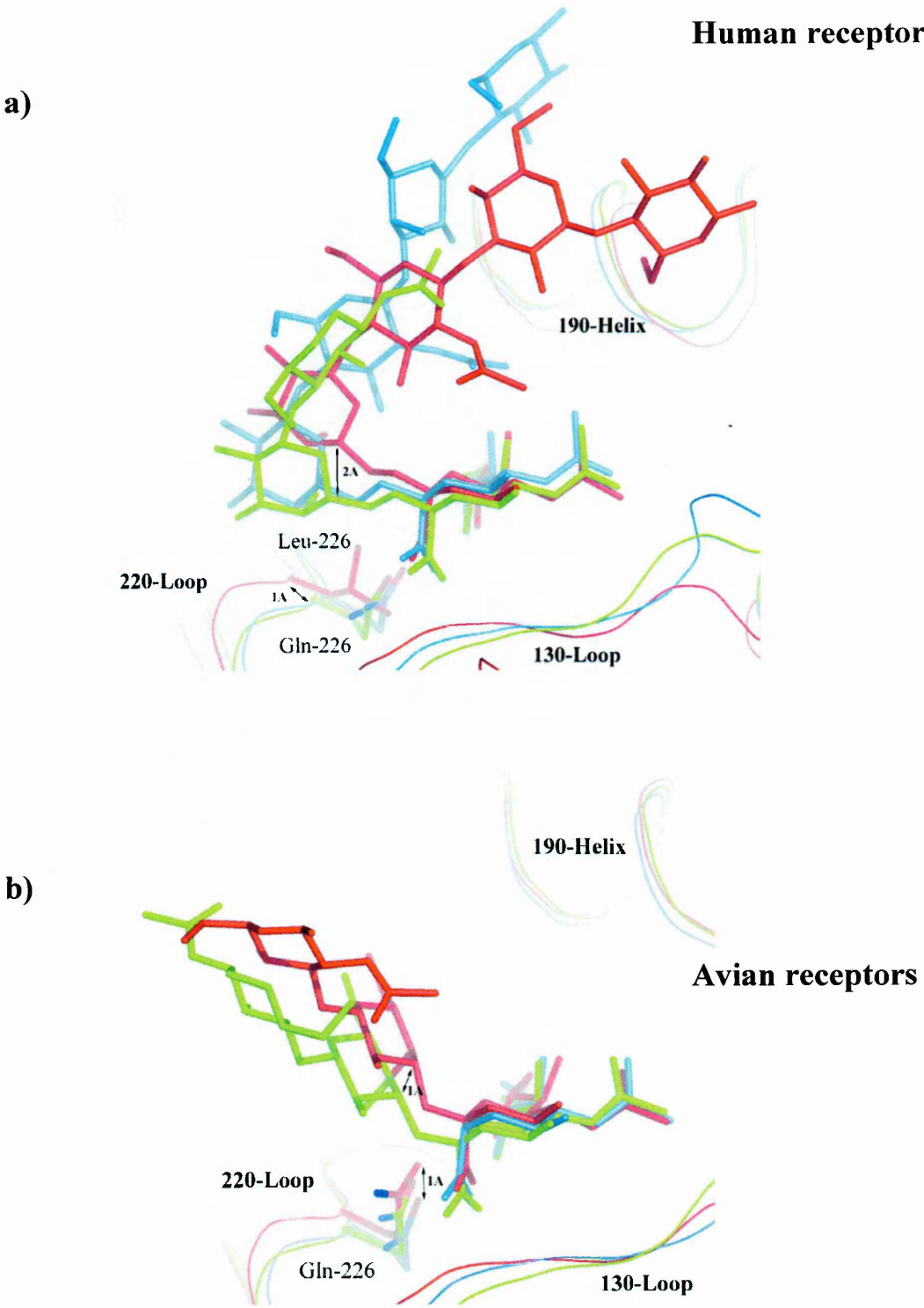


Figure 13 Overlay of receptor-binding sites of three different HAs with bound α(2,6)-linked receptors (a) and α(2,3)-linked receptors (b) Human H1 HA (Puerto Rico/8/34) is coloured in green, human H3 HA (X31) coloured in red and swine H1 HA (Sw/Iowa/15/30) coloured in blue. The receptor analogues are coloured according to the HA to which they are bound.

determined two different cell types in human tracheobronchial epithelium cultures, correlating with preferential infection by human and avian viruses. Ciliated cells contain mainly the α(2,3)-linkage and are infected by avian viruses, whereas non-ciliated cells predominant in the α(2,6)-linkage are infected by human viruses (Matrosovich et al., 2004). In contrast, intestinal cells of ducks, where infection is enteric (Kida et al., 1980), and tracheal cells of horses have been reported to be abundant in α(2,3)-linked sialic acid (Ito et al., 1998; Suzuki et al., 2000). Furthermore, swine respiratory tract cells contain both linkages, in agreement with the observation that viruses with either or "dual" linkage specificity have been isolated from pigs (Rogers and D'Souza, 1989; Gambaryan et al., 1997; Ito et al., 1998).

In addition to the linkage, the type of sialic acid recognised may also affect host restriction. The high amount of Neu5Gc in swine (50%) and horse (90%) tracheal epithelium compared to Neu5Ac has been shown to correlate with the ability of viruses isolated from these hosts to interact with this sialic acid species (Suzuki et al., 1997; Suzuki et al., 2000). The recognition of a minor fraction of Neu5Gc in duck intestine may also be important for replication in the duck intestine (Ito et al., 2000). By contrast, Neu5Gc has not been detected in normal adult human tissues (Schauer, 1982; Schauer, 1991).

Soluble sialylglycoconjugate inhibitors present in host serum or other body fluids might also contribute to the maintenance of virus receptor specificity. However, no formal proof is available for this hypothesis, which is based on the above described selection of linkage-specific variants by horse serum (Rogers et al., 1983b; Matrosovich et al., 1998), and the observation that mucins from the human lung contain sialic acid mainly in the α(2,3)-linkage (Breg et al., 1987; Baum and Paulson, 1990; Couceiro et al., 1993).

It should be noted that the HA-receptor interaction can also be influenced by the

length and composition of glycoproteins and glycolipids (Rogers and Paulson, 1983; Suzuki et al., 1986; Suzuki et al., 1992; Matrosovich et al., 1997; Ryan-Poirier et al., 1998; Gambaryan et al., 2005). This finding indicates that, in addition to sialic acid and its linkage to Gal, receptor-binding properties can be modulated by more distant parts of cellular receptors.

1.7.5 Antigenic Variation

In order to remain infectious and persist in the population, influenza virus needs to evade immune recognition. This is accomplished by antigenic variation in HA, which alters its antigenicity such that it is undetected by previously induced host defence systems. Antigenic variation is the main mechanism for the rapid evolution of influenza viruses and can be a result of two different processes, antigenic drift and shift.

1.7.5.1 Antigenic Shift

Antigenic shift is defined by the introduction of an HA to which the population contains little or no immunity. One mechanism through which antigenic shift can occur is reassortment of RNA segments between different viruses. Upon infection of a cell with two or more different viruses, hybrid progeny viruses can be packaged due to the segmented nature of their genome. First evidence for reassortment was provided by Burnet and Lind, who observed recombination of characters between viruses in mixed infections (Burnet and Lind, 1949). Reassortment between segments of different species origin, e.g. avian with human/equine/porcine, has been observed in tissue culture and laboratory animals, including turkeys and pigs (Tumova and Pereira, 1965; Webster et al., 1971). However, reassortment also occurs in nature (e.g. Castrucci et al., 1993; Gregory et al., 2002), and the introduction of novel HA subtypes into an immunologically naïve human

population can result in rapid global spread with widespread morbidity and mortality (pandemic). The Asian (1957) and Hong Kong (1968) pandemic have been caused by reassortant viruses containing an HA of avian origin (see *1.8 Emergence and Evolution of Human Influenza Viruses, p.61*).

In addition to reassortment, antigenic shift can occur by direct cross-species transmission of a virus containing a novel HA, e.g. sporadic infections of humans by avian viruses have been reported (Peiris et al., 1999; Fouchier et al., 2004). In particular, the avian H5N1 virus (bird flu), which is highly pathogenic for poultry, was first transmitted from chickens to humans in 1997 in Hong Kong (de Jong et al., 1997), resulting in 6 deaths of 18 identified cases (Bender et al., 1999). Although the source of infection was removed by slaughtering all the chickens and the virus has not been successfully spread from human to human, new cases of human infections are being reported since 2003, especially in Vietnam and Thailand. Due to its high mortality rate (57%) this virus poses a pandemic threat if it gains the ability to easily infect and transmit among people (WHO, 2005a).

The 1918 pandemic might also have been caused by direct transmission of an avian H1N1 virus (see *1.8 Emergence and Evolution of Human Influenza Viruses, p.61*).

1.7.5.2 Antigenic Drift

Antigenic drift is a consequence of accumulation of minor amino acid changes in HA, usually due to single point mutations. The rate at which these mutations occur is much higher for RNA viruses than for DNA viruses and eukaryotic organisms, since their RNA polymerase lacks proofreading activity and introduces errors in the range of 10^{-3}-10^{-4} per nucleotide, in contrast to 10^{-8}-10^{-11} for DNA polymerases (Holland et al., 1982). As a result of the fast mutation rate, the virus progeny consists of complex and dynamic mutant

distributions, also known as mutant swarms or mutant clouds, rather than of defined genomic sequences. RNA viruses have therefore been described as "quasispecies" (Domingo et al., 2001; Domingo, 2003). The influenza virus polymerase error-rate has been calculated to be approximately 1.5×10^{-5} per nucleotide per virus generation for the NS gene (Parvin et al., 1986). However, phylogenetic analysis has shown that HA of the H3 subtype human influenza viruses evolves 3 times faster than the non-structural proteins (Fitch et al., 1991). Furthermore, amino acid substitutions were observed to occur more frequently at antigenic than non-antigenic sites, suggesting positive Darwinian selection through immune surveillance. Subsequent studies are in support of this mechanism of evolution for both the H3 and H1 HA in the human population (Ina and Gojobori, 1994; Fitch et al., 1997; Bush et al., 1999b). Variants that escape neutralisation by antibodies exhibit a growth advantage and therefore have the potential to cause epidemics in the human population.

1.7.5.2.1 Antigenic Sites

Antigenic drift changes in influenza viruses have been monitored ever since their first isolation to ensure efficient vaccine production. These were initially detected by comparison of reactivity with convalescent ferret sera and later accompanied by nucleotide sequence analysis of the HA gene. After the first H3 HA was crystallised in 1981 (Wilson et al., 1981), these changes could be mapped on the structure and 5 distinct antigenic sites were proposed, designated A-E (Wiley et al., 1981; Daniels et al., 1983). However, it has now become clear that the majority of the HA_1 surface is antigenic. Between 1968 and 1997 more than 50% of the solvent-accessible amino acids in the membrane distal domain have been substituted (Fleury et al., 1998) (see Figure 14). Similar antigenic sites have been identified in the human H1 and H2, the avian H5 and the swine H9 subtypes and have

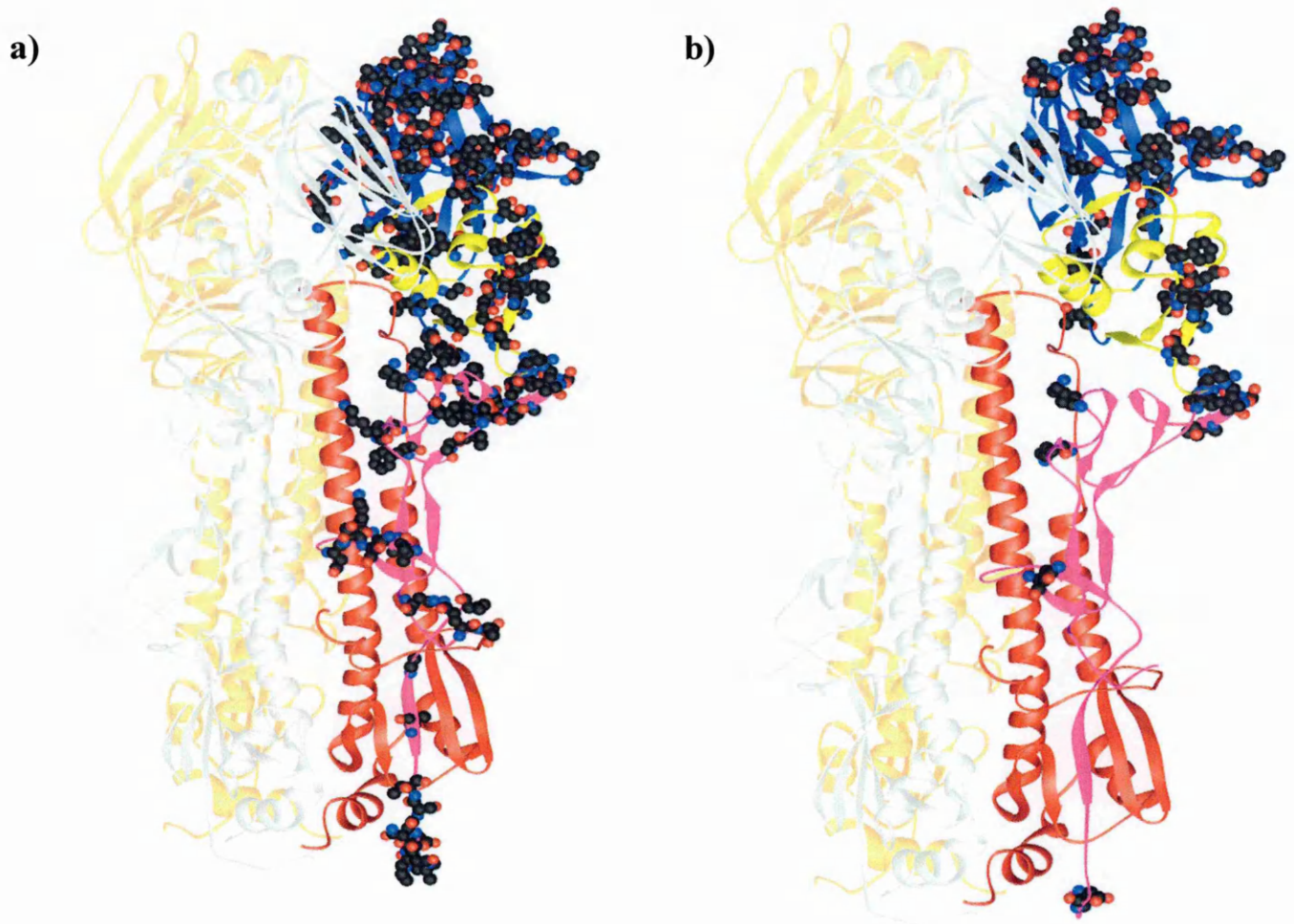


Figure 14 Amino acid changes in H3 HAs from 1968-2002 a) total number of substitutions at HA residues **b)** substitutions that became fixed during HA evolution. A BHA trimer is shown, with changes indicated on one monomer. The HA_1 domain is coloured in magenta/yellow/blue, the HA_2 domain in red. Circles are constituents of amino acids (red = oxygen atoms, blue = nitrogen atoms, black = carbon atoms).

been mapped on their solved structures (H5 and H9) or on the H3 structure (H1 and H2) (Caton et al., 1982; Tsuchiya et al., 2001; Kaverin et al., 2002; Kaverin et al., 2004).

The involvement of these sites in antibody recognition is strongly supported by the observation that amino acid substitutions of monoclonal antibody (mAb)-selected antigenic variants are located in these sites and often coincide with substitutions found in natural variants (Wiley et al., 1981). It was also suggested that substitutions in these sites would only cause local structural changes in HA without affecting the main framework of the molecule or receptor-binding activity. X-ray studies of mAb-selected mutant HAs have confirmed that escape from neutralisation is accompanied by only small structural changes in the immediate vicinity of the amino acid substitution, suggesting binding of the antibodies to that area (Knossow et al., 1984; Sauter et al., 1992; Bizebard et al., 1995; Fleury et al., 1998). Direct evidence for the interaction of antibodies with these sites was provided by electron microscopy studies of HA complexed with mAbs recognising different epitopes (Wrigley et al., 1983) and subsequently confirmed by X-ray studies of complexes of monoclonal Fabs with HA. These studies also showed that selected mutations, which allow the virus to escape neutralisation, are located in the epitopes (Bizebard et al., 1995; Fleury et al., 1998; Fleury et al., 1999).

Protruding loops as antibody-binding site motifs have also been determined for influenza NA (Colman et al., 1983), picornaviruses (Hogle et al., 1985; Rossmann et al., 1985) and have also been suggested for HIV-1 gp120 (Wyatt et al., 1998).

1.7.5.2.2 The Role of Oligosaccharides in Antigenic Drift

Immune-evasion can also be mediated by oligosaccharides protruding from the HA surface. These have the potential to mask the HA surface as antigenically "self" because of their host cell origin and can inhibit antibody binding by steric hindrance. Shielding of

functional epitopes has been directly shown by studies with viruses containing additional carbohydrates (Skehel et al., 1984; Gallagher et al., 1988; Munk et al., 1992; Abe et al., 2004). Support for the involvement of oligosaccharides in immune-evasion is provided by antigenic drift mutations that create new oligosaccharide attachment sites (e.g. Seidel et al., 1991). The 1968 influenza H3 viruses contained six attachment sites at HA_1 residues 8, 22, 38, 81, 165 and 285, with the last three located at the membrane-distal part of the molecule (Wilson et al., 1981). Since then, additional oligosaccharide attachment sites were accumulated and the one at residue 81 was lost. The majority of viruses isolated since 1999 contain a total number of 11 carbohydrate sites (at residues 8, 22, 38, 63, 122, 126, 133 144, 165, 246 and 285), eight of which are located in antigenically important regions. A similar increase of oligosaccharide attachment sites has been shown for the H1 subtype from 1918-2002. The 1918 pandemic H1 virus contained 4 attachment sites (at residues 21, 33, 94a and 289) (Gamblin et al., 2004), whereas further attachment sites were accumulated in subsequent years. Therefore, viruses since 1999 usually contain additional glycosylation sequons at residues 63, 129 and 163 (WHO, 2002a).

Implication of oligosaccharides in immune-evasion has also been suggested for HIV-1 and SIV-1 virus surface glycoproteins (Schonning et al., 1996; Reitter et al., 1998).

1.7.5.2.3 Antibody-Binding and Mechanism of Neutralisation of Infectivity

Structural studies have also addressed the molecular mechanisms involved in antibody escape and virus neutralisation. Fleury *et al.* have shown that a Fab bound to an epitope in the immediate vicinity of the RBS abrogates virus-cell binding, because it contacts three conserved residues that interact with sialic acid (Fleury et al., 1999). Neutralisation was also observed when the antibody was bound to epitopes located further from the RBS, in this case mediated by steric hindrance with the bulk of either the Fab or immunoglobulin

molecule. However, efficiency of neutralisation correlates with avidity of the antibody for HA, and it has been shown that the most potent inhibitors are antibodies that directly block the RBS (Fleury et al., 1999).

Escape from neutralisation is associated with a significant decrease in affinity of the selecting antibody for mutant HAs by a factor of 10^2-10^4 compared to a K_D of ~ 10^{-9} M for wild-type HA (Fleury et al., 1998). The reduced affinity appears to be mediated by steric hindrance in the antigen-antibody interface as a result of the local structural changes in the site (Knossow et al., 1984; Bizebard et al., 1995). Another mechanism, antigen distortion, has been implicated in inhibition of binding to antibodies. It has been shown that the Thr131Ile HA mutant contacts the antibody in a contorted wild-type-like conformation, leading to the break of a hydrogen bond in the interaction interface (Fleury et al., 1998). Therefore, escape from neutralisation can be mediated by both steric hindrance and/or the loss of one or more interactions (hydrogen bond, salt bridge) between HA and antibody.

1.7.6 Interrelationship between Antigenic Variation and Receptor-Binding Properties

The variable antigenic sites in HA are all clustered around the highly conserved RBS. In order to maintain infectivity of the virus, there is a constraint under which the mechanism for escape from neutralising antibodies operates: Antigenic variation must occur while conserving residues involved in receptor-binding activity. This criterion is fulfilled due to a greater surface area of the "antibody-footprint" compared to the area of the receptor-binding pocket (600-1,000 $Å^2$ versus 315 $Å^2$). This ensures that all HA-antibody interfaces include HA residues not required for receptor-binding function (Bizebard et al., 1995). Changes in these functionally non-important residues is therefore considered sufficient to block binding to antibody. The same observation has been made for influenza NA

(Colman, 1997) and rhinovirus (Smith et al., 1996). However, it has been shown that changes in antigenicity can be accompanied by differences in receptor-binding or growth properties and vice versa. Direct evidence for a concomitant change in these properties has been reported for changes at residues located in the RBS (Rogers et al., 1983a; Daniels et al., 1984; Naeve et al., 1984; Martin et al., 1998), in antigenic sites in the vicinity of the RBS (Both et al., 1983a; Underwood et al., 1987; Smith et al., 1991) or in combination (Laeeq et al., 1997). Furthermore, substitutions at residues in antigenic sites distant from the RBS have also been shown to influence both antigenicity and interaction with cellular receptors. This finding was observed for amino acids located in the HA trimer interface (Daniels et al., 1987; Suzuki et al., 1989) or for residues involved in creating or abolishing glycosylation sites (e.g. Skehel et al., 1984; Robertson et al., 1985). In addition to their implication in resistance to antibody-recognition (see above), the number and position of oligosaccharides at the membrane-distal part of HA have been shown to influence receptor-binding properties by providing steric hindrance with receptors (Deom et al., 1986; Gunther et al., 1993; Matrosovich et al., 1997; Ohuchi et al., 1997; Abe et al., 2004). Furthermore, the glycosylation pattern, which is determined by the host cell, can also influence receptor-binding properties (Crecelius et al., 1984; Deom et al., 1986; Aytay and Schulze, 1991; Mir-Shekari et al., 1997; Gambaryan et al., 1998; Romanova et al., 2003).

Importantly, viruses with altered receptor-binding properties have been selected *in vitro* with monoclonal or polyclonal antibodies. Many of these, containing substitutions at residues close to or in the RBS, are not antigenic variants, since they still interact with the selecting antibody in enzyme-linked immunosorbent assays (ELISA). They therefore qualify as adsorptive mutants. These have been reported to display a general increase in affinity for cellular receptors as judged by binding to periodate- or NA-treated RBC (Fazekas de St. Groth, 1977; Yewdell et al., 1986; Underwood et al., 1987). In contrast to

NA, which cleaves off the entire molecule, periodate destroys the glycerol chain of sialic acid. In more detailed studies differences in affinity of such variants for receptor analogues containing either α(2,3)- or α(2,6)-linked sialic acid were observed. These included increased affinity for the α(2,3)-linkage with or without decrease in affinity for the α(2,6)-linkage (e.g. Gly218Glu, Ser193Arg, Ser193Asn/Leu226Pro, 224-230 deletion) (Daniels et al., 1987), decreased affinity for the α(2,3)-linkage (Ser193Ile, Ser205Tyr) (Daniels et al., 1987; Suzuki et al., 1989), decrease in affinity for the α(2,6)-linkage (Glu190Asp/Leu226Gln) (Temoltzin-Palacios and Thomas, 1994) and decrease in affinity for both linkages (e.g. Gly218Arg, Gly135Arg/Gly225Asp, Ser145Asn/Leu226Gln) (Daniels et al., 1987; Laeeq et al., 1997). The implications of the selection of receptor-binding variants by antibodies for immune-evasion are discussed in *4.1.2 Molecular Mechanism for Antigenic Drift, p.132.*

In summary, all the studies described above show that antigenic and receptor-binding properties can be interrelated. This observation is most likely due to the close proximity of antigenic sites to the RBS. Residues located in an epitope have the potential to affect receptor-binding properties if they directly contact an amino acid involved in receptor-recognition by affecting its position. Likewise, a change at a RBS residue could influence the position of a neighbouring amino acid making part of an epitope and therefore affect antigenicity. Substitutions in antigenic sites located further away from the RBS can influence the interaction with cellular receptors if they create or abolish a glycosylation site (see above). Furthermore, it has been shown that changes at residues in the HA interface also have the potential to affect receptor-binding properties, probably by altering the quaternary structure of HA (Daniels et al., 1987; Suzuki et al., 1989).

1.8 Emergence and Evolution of Human Influenza Viruses

1.8.1 Origin of Pandemic Influenza Viruses

Only 3 of the 16 HA subtypes circulating in waterfowl have evolved in the human population. The emergence of each of these has caused a major pandemic in the last century: H1 in 1918 (Spanish flu), H2 in 1957 (Asian pandemic), H3 in 1968 (Hong Kong pandemic). The appearance of every new subtype has led to the replacement of the previous one, with the exception of the re-introduction of the H1 subtype in 1977 (Russian flu). Since then, H1 and H3 subtypes co-circulate in the human population and cause recurrent epidemics due to antigenic drift in HA. Including reports of the sequential emergence of H2 viruses in 1889 and H3 viruses in 1900, these 3 subtypes appear to recycle through the human population in a cyclical pattern (Masurel and Marine, 1973).

The H1N1 subtype introduced into the human population in 1918 appears to be avian-like, based on phylogenetic analysis (Gorman et al., 1990a; Gorman et al., 1991) and direct sequencing of the HA isolated from 5 people who died in the 1918 pandemic (Taubenberger et al., 1997; Reid et al., 1999; Reid et al., 2003). However, due to the limited number of available samples, little is known about the identity of the H1N1 viruses circulating before and around 1918. Furthermore, the human H1N1 subtype appears to share its common avian ancestor with the classic swine virus, which was introduced into pigs around the same time (Gorman et al., 1991). Therefore, it remains elusive whether the pandemic 1918 virus was transmitted directly from birds, or whether the virus circulated in a pre-pandemic period in humans, pigs or other species with the potential to mutate and/or reassort.

The 1957 pandemic strain resulted from reassortment of a human virus with a co-circulating avian virus, leading to the introduction of avian HA, NA and PB1. In 1968, the H2 HA and PB1 were again replaced by avian-derived segments, generating the new

H3N2 subtype (Webster and Laver, 1972; Scholtissek et al., 1978; Kawaoka et al., 1989). The identity of avian strains with which the human viruses reassorted has not been identified. However, sequence, antigenic and phylogenetic analysis of HA of the 1957 and 1968 pandemic strains suggests the avian progenitor viruses to be Eurasian-like and Dk/Ukraine/1/63-like, respectively (Laver and Webster, 1973; Fang et al., 1981; Bean et al., 1992; Schafer et al., 1993). The host for the generation of the 1957 and 1968 reassortants has not been identified. The pig has been suggested to be a likely candidate to serve as a "mixing vessel" for human and avian viruses (Scholtissek et al., 1985). It has been shown that a variety of human and avian viruses can replicate in pigs upon experimental infection (Hinshaw et al., 1981; Kida et al., 1994). Transmission from birds and humans to pigs has also been observed in a natural environment. Notably, the swine-endemic H1 and H3 subtypes are either of avian (classic and avian-like H1N1 viruses) or of human (human-like H3N2 viruses) origin (Ottis et al., 1982; Gorman et al., 1991; Schultz et al., 1991). However, although avian and human viruses have reassorted in pigs (Castrucci et al., 1993; Brown et al., 1998), transmission of such a virus to humans has only been reported once (Claas et al., 1994). Alternatively, chickens have been proposed as intermediate hosts for the generation of pandemic viruses (Matrosovich et al., 2001; Gambaryan et al., 2002; Gambaryan et al., 2003), and the possibility of reassortment of human and avian viruses in the human lung has also been suggested (Matrosovich et al., 2004).

1.8.2 Early Changes in Haemagglutinin in Adaptation to the Human Host

The molecular mechanism by which avian viruses adapt to growth in humans is poorly understood. However, interaction with human receptors is believed to be an initial requirement for introduction of avian-like HA into humans (Ito et al., 1998; Matrosovich et

al., 2000). Indeed, viruses isolated at the beginning of the 1957 and 1968 pandemics have acquired the ability to bind to α(2,6)-linked receptors (Matrosovich et al., 2000). This early change in receptor-binding properties was attributed mainly to the Gln226Leu substitution compared to avian isolates. In contrast, since no crystal structures of avian H1 HAs are available, it is not clear whether structural changes in the RBS were required for the initial transfer of H1N1 viruses from birds to humans. However, receptor-binding studies with a reassortant virus containing the HA of a human 1918 isolate indicate that the earliest human H1 viruses exhibited clear α(2,6)-linkage preference typical of human isolates (Kobasa et al., 2004). In addition, the HA structure of the 1918 HA is very similar to that of another early human H1 (A/Puerto Rico/8/34) and a closely related swine (Sw/Iowa/15/30) strain, both of which were found in complex with α(2,6)-linked receptor analogues (Gamblin et al., 2004). This finding supports the ability of the 1918 virus to bind to the α(2,6)-linkage.

Upon transfer to the human population, a shift in linkage recognition from α(2,3) to α(2,6) might represent adaptation to the new host. It was observed that H1 viruses from the first period 1918-1957 often recognised both avian and human receptors, whereas viruses appear to be more human receptor-specific upon re-emergence in 1977 (Rogers and D'Souza, 1989). Substitutions Glu190Asp and Gly225Asp in early H1 human isolates have been suggested to play a role in an increase of affinity for α(2,6)-linked receptors (Matrosovich et al., 2000), whereas loss of ability to bind to α(2,3)-linked receptors was proposed to be due to Ala138Ser in later strains (Rogers and D'Souza, 1989). Similarly, whereas early H3 viruses were able to bind to short α(2,3)-linked gangliosides, this property was gradually lost upon circulation in humans and correlated with the acquisition of a new glycosylation site at position 126 (Matrosovich et al., 1997). The substitution Gly228Ser has also been shown to reduce affinity towards α(2,3)-linked receptors

(Matrosovich et al., 2000), which might be advantageous in providing increased resistance to α(2,3)-linkage-containing inhibitors in the human lung. The positions at which these early substitutions occurred are highly conserved in the avian RBS of HA subtypes H1-15 (Ala138, Glu190, Leu194, Gly225, Gln226 and Gly228), providing further evidence for their implication in interspecies transmission (Matrosovich et al., 1997).

In conclusion, all the introduced avian HAs share the common feature of recognition of the α(2,6)-linkage. The failure of the 1997 avian H5N1 virus to spread among the human population might have been a result of the inability of these viruses to recognise the human receptors (Matrosovich et al., 1999).

Since their introduction into the human population, HA and NA have been and are still evolving, because they are subject to strong selection pressure by antibodies (see *1.7.4.4 Host Cell Factors Involved in Selection of Linkage Specificity, p.49).* Evolution of these proteins is also observed in pigs (Brown, 2000). In contrast, the evolutionary rate of influenza proteins in birds is very slow and they therefore appear to be in evolutionary stasis (Kida et al., 1987; Gorman et al., 1990a; Gorman et al., 1990b; Bean et al., 1992). This might be due to poor immune pressure, since influenza viruses do not cause disease in aquatic birds (Kida et al., 1980). The avirulent nature of avian influenza may therefore reflect an optimal adaptation to this host. Alternatively, the slow evolution of influenza virus might be due to the relatively short life-span of birds compared to humans. These animals might simply not live long enough to become re-infected by the same virus strain, accounting for the lack of selection pressure (Kida et al., 1987). Perpetuation of all 16 HA subtypes in aquatic birds provides a reservoir for the potential introduction of novel HA subtypes into other species.

1.9 Structure and Function of Neuraminidase

NA plays important roles in the virus life cycle. It cleaves the receptor sialic acid from the host cell surface upon virus assembly at the plasma membrane, facilitating virus release and therefore spread of infection (see *1.4 The Influenza Virus Life Cycle, p.22*). In addition the enzyme removes sialic acid from oligosaccharides on HA and NA (Basak et al., 1985), preventing-self aggregation of virus and promoting haemagglutination activity (Ohuchi et al., 1995). Evidence for these functions has been provided by studies using anti-NA antibodies (Compans et al., 1969), NA inhibitors (Palese et al., 1974a; Gubareva et al., 1996) and viruses containing impaired or no NA activity (Palese et al., 1974b; Liu and Air, 1993). All these studies observed the formation of large clusters of aggregated virus at the cell surface due to NA impairment. Furthermore, NA has the ability to inactivate certain sialic acid-containing HA-inhibitors (Francis, 1947). Therefore, activity of this enzyme might facilitate access to epithelial cells by degradation of inhibitory sialic acid-containing mucins in the respiratory tract (Burnet, 1951).

Like HA, NA is an antigenic determinant and is subject to antigenic variation, although antibodies do not have neutralising activity (Kilbourne et al., 1968). Nine different non-cross reactive subtypes are found in nature (N1-N9), of which N1 and N2 circulate in humans (Schild et al., 1980).

The three-dimensional structure of NA has been solved and revealed the catalytic and antigenic sites (Colman et al., 1983). NA is a glycosylated tetramer of ~ 240,000 Da, consisting of a box-shaped globular head attached to a thin stalk spanning the virus membrane. Neu5Ac binds into a large pocket on the distal surface surrounded by conserved amino acids, which is, as in HA, flanked by antibody binding sites. Knowledge of the structure served as a basis for the development of potent, highly specific inhibitors of NA activity. Two of these, oseltamivir (GS4104, Tamiflu) (Kim et al., 1997) and

zanamivir (GG167, Relenza) (von Itzstein et al., 1993) are now approved for therapeutic use in humans, and no resistance to these drugs has been reported in circulating viruses so far. However, resistant variants have been selected upon repeated passaging of virus in cell culture in the presence of these inhibitors and have been identified clinically (Gubareva et al., 2000).

Similarly to HA, NA shows distinct recognition of different sialic acid species and their linkage to Gal, which is dependent on the viral isolate examined. Since its introduction from an avian virus, the human N2 has changed its specificity over the years. Earliest isolates showed activity only for the α(2,3)-linkage typical for avian isolates (Carroll et al., 1981; Corfield et al., 1982; Corfield et al., 1983), followed by a gradual drift towards equal recognition of the α(2,3) and α(2,6)-linkage (Baum and Paulson, 1991). This finding indicates the development towards the same specificities of HA and NA as a result of evolution in hosts. Likewise, in correlation with their ability to bind to Neu5Gc (Suzuki et al., 1997), NA from swine viruses has also shown activity towards the N-glycolyl species of sialic acid (Xu et al., 1995).

1.10 Functional Balance between HA and NA Activity

Due to the antagonistic nature of HA and NA, the receptor-binding and receptor-destroying activities and specificities need to be carefully balanced for optimal virus replication. Evidence for a functional interplay between HA and NA has been provided by a number of studies. As described above, the specificity of NA and HA towards the species of sialic acid and its linkage in the receptor have been shown to correlate. Studies with NA-deficient viruses have shown that a lack of NA activity can be partially compensated for by a decrease of HA affinity towards cellular receptors (Hughes et al., 2000). Sequence analysis of HA revealed substitutions around the receptor-binding pocket (e.g. Ser193Arg,

Val205Met; Gly135Ala, Ser145Asn, Arg220Lys). A decrease in HA affinity was also determined by studies with reassortant viruses displaying an HA-NA mismatch caused by the inability of NA to completely desialylate HA. Accompanying substitutions in the proximity of the RBS were often of negative charge (e.g. Asn248Asp). Therefore, a mechanism by which electrostatic repulsion between negative charges of HA and sialic acid causes a decrease of affinity was postulated (Kaverin et al., 1998; Kaverin et al., 2000). The importance of functional interplay between HA and NA was also highlighted in studies on genetically engineered viruses. Viruses generated by reverse genetics containing a shortened stalk or no stalk showed a markedly decreased efficiency of virus replication in eggs, in correlation with a reduced ability of these viruses to elute from RBC. Steric hindrance due to the location of the catalytic site too close to the viral envelope was suggested to impair NA function (Castrucci and Kawaoka, 1993). Adaptation of these mutants to growth in eggs led to compensating mutations in HA with a decreased affinity towards sialylglycoconjugates (Mitnaul et al., 2000). In contrast, deletions of N-glycan attachment sites at the tip of HA increased the affinity towards cellular receptors (Ohuchi et al., 1997). Growth of viruses carrying the HA glycosylation mutations was greatly impaired when combined with a low activity-reduced stalk N1 NA (Wagner et al., 2000). This interdependence of HA glycosylation and NA stalk length was confirmed in a later study (Baigent and McCauley, 2001). In particular, combinations of either an HA glycosylated at residue 158 with a short-stalk NA or HA lacking this glycosylation site with a full-length stalk NA led to optimal virus replication. Similar concerted changes in HA and NA stalk length have also been observed under natural conditions, e.g. transmission of viruses from wild aquatic birds to domestic land-based poultry was accompanied by deletions in the NA stalk and introduction of an additional N-linked glycan in HA_1 (Matrosovich et al., 1999; Banks et al., 2001). Thus, concomitant changes

might be implicated in adaptation of influenza virus to a new host. Finally, compensating mutations in HA have also been observed in NA-inhibitor resistant viruses and present an alternative mechanism of drug resistance to mutations in the NA catalytic site (McKimm-Breschkin et al., 1996; Bantia et al., 1998; Blick et al., 1998). Due to their decreased affinity for cellular receptors, elution of these variants is less dependent on NA activity and can therefore occur in the presence of inhibitor.

1.11 Objectives

The aim of this investigation was to elucidate further the interrelationship between antigenic variation and receptor-binding properties of HA. Antigenic variants of the H3 and H1 subtypes were identified by probing with convalescent ferret sera and sequence comparison from the time of the first appearance of these viruses in humans until the present. A sensitive microscale SPR assay using the BIAcore biosensor system was established to identify differences in receptor-binding properties of these viruses. Monitoring changes in affinity and/or specificity for receptor analogues would complement surveillance studies and provide additional information on the evolution of influenza virus in the human host.

In addition, it has previously been possible to select for an avian virus preferentially recognising the sialic acid linkage typical for human viruses (Rogers et al., 1985). This alteration in receptor-binding specificity was accompanied by a mutation Gln226Leu, which, however, reverted upon growth of the variant in eggs. These selection experiments were repeated with an additional avian virus with the aim to identify the substitutions involved in the switch from preferential receptor recognition from avian-to human-like.

2 Materials and Methods

All reagents were obtained from BDH Laboratory Supplies (Merck, UK), except where specified.

2.1 Standard Buffers and Solutions

2.1.1 Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

2.1.1.1 SDS-Polyacrylamide Gel (non-reducing)

Resolving Gel (12%)

0.4 M Tris-Hydroxymethylaminoethane (Tris) /HCl pH 8.9

12% (w/v) acrylamide/Bis 37.5:1 solution (BioRad)

0.1% (w/v) SDS (BioRad)

0.01% (v/v) N,N,N',N'-Tetramethylethylenediamine (TEMED) (Sigma)

0.1% ammonium persulphate (APS) (w/v) (BioRad)

Stacking Gel (6%)

60 mM Tris/HCl pH 6.7

6% (w/v) acrylamide/Bis 37.5:1 solution (BioRad)

0.1% (w/v) SDS (BioRad)

0.01% (v/v) TEMED (Sigma)

0.1% (w/v) APS (BioRad)

2.1.1.2 SDS-Gel Loading Buffer 5 x (non-reducing)

62.5 mM Tris/HCl pH 6.8

10% (v/v) glycerol

2% (w/v) SDS (BioRad)

Bromophenol Blue (Sigma)

2.1.1.3 SDS-Gel Running Buffer 10 x

0.248 M Tris

1.92 M glycine

1% (w/v) SDS (BioRad)

pH 8.3

2.1.1.4 Coomassie Stain Solution for SDS-Gels

40% (v/v) methanol

10% (v/v) acetic acid

0.25% (w/v) Coomassie Blue (BioRad)

2.1.1.5 Destaining Solution for SDS-Gels

40% (v/v) methanol

10% (v/v) acetic acid

2.1.2 Agarose Gels

2.1.2.1 10 x Tris Borate/EDTA (TBE)

(prepared by NIMR media services)

0.9 M Tris

0.9 M boric acid

20 mM ethylenediaminetetraacetic acid (EDTA)

pH adjusted to 8.4

2.1.2.2 Agarose DNA Gel Loading Buffer

30% (w/v) sucrose

Bromophenol Blue (Sigma)

2.1.3 Sequencing

2.1.3.1 Loading Buffer for Sequencing

95% formamide (Sigma)

10 mg/ml Blue Dextran (Pharmacia)

5 mM EDTA

pH adjusted to 8.0

2.1.4 Primers

Oligonucleotide primers for reverse transcription, PCR and sequencing were supplied by Oswel:

Reverse Transcription:

Uni12	5'	-AGC AAA AGC AGG-	3'

H3N2 viruses:

H3HAF6	5'	-AAG CAG GGG ATA ATT CTA TTA ACC-	3'
H3A1R1	5'	-GTC TAT CAT TCC CTC CCA ACC ATT-	3'
H3HAF567	5'	-CTG AAC GTG ACT ATG CCA AAC AAT-	3'

H3HAR650	5'	-TTG GTC ACT GTC CGT ACT CGG GTG-	3'

H1N1 viruses:

H1A1F1	5'	-CAA CCA AAA TGA AAG CAA AAC TAC-	3'
H1A1R1/2	5'	-ATA CCA CCC ATC CAT CAT TCC A-	3'
H1F594	5'	-GAA GTC CTT GTA CTA TGG GGT GTT-	3'
H1R623	5'	-GAT GAA CAC CCC ATA GTA CAA GGA-	3'
H1R1036	5'	-CTT AGT CCT GTA ACC ATC CT-	3'
H1R1087	5'	-AAA CCG GCA ATG GCT CCA AA-	3'
SPHAF1	5'	-AGC AAA AGC AGG GGA AAA TA-	3'
SPHAR13	5'	-TAC CAT CCA TCT ATC ATT CCA GTC CA-	3'

2.1.5 Cell Culture

2.1.5.1 Complete Medium for Cell Culture

Dulbecco's modified eagle's medium containing pyridoxine (DMEM, Gibco)

5% (v/v) heat-inactivated (56°C, 2 hours) fetal calf serum (FCS, PAA Laboratories, GMbH)

100 U/ml penicillin, 0.1 mg/ml streptomycin (Sigma)

2.1.5.2 Trypsin Versene

(Supplied by NIMR media services)

0.8% (w/v) NaCl

0.02% (w/v) KCl

0.12% (w/v) Na_2HPO_4

0.02% (w/v) KH_2PO_4

0.01% (w/v) EDTA

0.13% (w/v) trypsin

0.001% (w/v) phenol red

pH adjusted to 7.8

2.1.5.3 Plaque Assay

Overlay Medium

45 ml 2x DMEM (Gibco)

2 ml 8 mg/ml Diethylaminoethyl-Dextran (Pharmacia)

1 ml 100x non-essential amino acids (Gibco)

1 ml penicillin (10,000 U/ml)/streptomycin (10 mg/ml) (Sigma)

1 ml 0.2 M glutamine (Gibco)

0.1 ml 2.5 mg/ml L-(tosylamido-2-phenyl) ethyl chloromethyl ketone

(TPCK)-treated trypsin (Sigma)

50 ml 1.5% noble agar (prepared by NIMR media services), melted in boiling water bath

The mixture was cooled down to 37°C before use.

2.1.6 Reagents for Neuraminidase Assay

2.1.6.1 Periodate Solution

0.2 M sodium periodate (meta)

9 M orthophosphoric acid

2.1.6.2 Arsenite Solution

0.77 M sodium m-arsenite (Sigma)

0.5 M sodium sulphate (anhydrous)

0.05 M sulfuric acid

2.1.6.3 Thiobarbituric Acid

0.04 M thiobarbituric acid (Sigma)

0.5 M sodium sulphate (anhydrous)

2.1.6.4 Complete Fetuin Substrate

Fetuin substrate was prepared by ammonium sulphate precipitation: 1 L 5.75 M ammonium sulphate was added to 1 L fetal calf serum (PAA Laboratories) at 4°C. After centrifugation at 2,000 g (1 hour, 4°C) in a Beckman J6-MC centrifuge, the precipitate was dialysed in Visking dialysis tubing (Medicell International Ltd.) against distilled water with buffer changes until no more precipitate was visible.

2.1.7 Other Buffers

2.1.7.1 Phosphate-Buffered Saline (PBS)

(prepared by NIMR media services)

1% (w/v) NaCl

0.025% (w/v) KCl

0.14% (w/v) Na_2HPO_4

0.025% (w/v) KH_2PO_4

2.1.7.2 Desialylation Buffer

10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (Sigma)

150 mM NaCl

10 mM $CaCl_2$

0.5 mM $MgCl_2$

pH adjusted to 6.0

2.1.7.3 Resialylation Buffer (HEPES buffer)

10 mM HEPES (Sigma)

150 mM NaCl

10 mM $CaCl_2$

0.5 mM $MgCl_2$

1 mg/ml D (+) glucose

5 mg/ml BSA (Sigma)

pH adjusted to 7.4

2.1.7.4 HBS-EP Buffer

(supplied by BIAcore)

0.01 M HEPES pH 7.4

0.15 M NaCl

3 mM EDTA

0.005% (v/v) Surfactant P20

2.2 Methods

2.2.1 Molecular Biology Techniques

2.2.1.1 Viral RNA extraction

Viral RNA extraction was performed using the QIAamp Viral RNA mini spin kit (QIAgen) according to manufacturer's instructions. Virus grown in cell culture, eggs (allantoic fluid or purified virus) or resuspended from picked plaques were used to isolate RNA.

2.2.1.2 Reverse Transcriptase PCR (RT-PCR)

cDNA was synthesised from vRNA using AMV reverse transcriptase (Roche) using a PTC-100 Programmable Thermal Controller (MJ Research, Inc.). The reaction mixture consisted of 5 µl viral RNA, 1 x incubation buffer, 1 mM deoxynucleotidetrisphosphates (dNTP) (Amersham Pharmacia), 100 ng primer (Oswel) and 12.5 U reverse transcriptase made up to 10 µl with double distilled water. The reaction was incubated at 42°C for 1 hour, 95°C for 10 minutes (min) and then cooled down to 4°C.

2.2.1.3 Polymerase Chain Reaction (PCR)

cDNA was amplified on a PTC-100 Programmable Thermal Controller (MJ Research, Inc.). A reaction mixture was prepared containing 5 µl of the RT-PCR product, 1 x incubation buffer, 0.2 mM dNTP (Amersham Pharmacia), 100 ng of forward and reverse primer, 2.5 U Cloned Pfu DNA polymerase (Stratagene) made up to 50 µl with double distilled water. The DNA amplification program consisted of 35 cycles of a denaturation step for 15 seconds (sec) at 95°C, an annealing step for 15 sec at 50°C followed by an elongation step for 2 min at 72°C. An additional elongation step was performed for 10 min

at 72°C to complete any partly synthesised DNA strands and then the reaction mixture was kept at 4°C.

2.2.1.4 Purification of PCR Products

Gel-extracted DNA fragments were purified using the QIAquick Gel Extraction kit (QIAgen) according to manufacturer's instructions.

2.2.1.5 DNA Sequencing

DNA was labelled with dye-terminators using the BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems). Approximately 50 ng of PCR product as determined by agarose gel electrophoresis using 50 ng of pGEM (Applied Biosystems) as a marker were added to the reaction mix (4 µl Big Dye reagent, 4 µl Half term diluent, 3.2 pmoles sequencing primer) and diluted to 20 µl with double distilled water. Following the sequencing programme (25 cycles of 96°C for 30 sec, 50°C for 15 sec, 60°C for 4 min, then cooling down the reaction mixture to 4°C) the DNA was precipitated. The reactions were mixed with 50 µl 96% ethanol and 2 µl 3 M sodium acetate pH 5.2, and incubated on ice for 10 min. The DNA was pelleted at 10,000 g for 15 min in an Eppendorf 5415 C centrifuge. The pellet was washed in 500 µl 70% ethanol, respun at 10,000 g for 5 min and dried on a heating block at 90°C for 1 min. The DNA was resuspended in 4 µl loading buffer and run on the ABI PRISM 377 DNA sequencer (Applied Biosystems) according to the manufacturer's instructions. Sequence data were assembled and edited using the Staden Package Version 1.5.3 (2004.1). Sequence data were analysed using the Wisconsin Sequence Analysis Package Version 10 (GCG) and phylogenetic trees were constructed using PAUP (Phylogenetic Analysis Using Parsimony, Version 4.0, D. Swofford, Illinois Natural History Survey, Champaign, IL, USA) (Swofford and Olsen, 1990).

2.2.1.6 Agarose Gel Electrophoresis

Samples were mixed with 10 x loading buffer and separated on 1% agarose (BioRad) gels in 1 x TBE containing 0.5 μg/ml ethidium bromide (Sigma). Electrophoresis was carried out at 80 V, limiting to 150 mA. DNA bands were visualized by trans UV illumination and digital images were captured with a Kodak Image station 440 CF and Digital Science 1D software (Perkin Elmer). A 1 kb DNA ladder (12216-298 bp, Gibco) was used as DNA size marker.

2.2.2 Biochemical and Cell Biology Techniques

2.2.2.1 SDS-PAGE

SDS-PAGE gels containing a 6% stacking and a 12% resolving gel were run using a EC120-4 Mini-Vertical Gel System (Thermo Electron Corporation). Samples were mixed with 5 x loading buffer and denatured on a heating block at 90°C for 5 min. The proteins were separated by electrophoresis in SDS gel running buffer at 200 V, limiting to 50 mA per gel. The gels were stained in Commassie Blue stain for 20 min and destained overnight in destain solution. Kaleidoscope Prestained Standards (210,000-7,200 Da, BioRad) were used as protein size markers.

2.2.2.2 Western Blotting

Proteins resolved by SDS-PAGE were transferred to Immobilon-P membranes (Millipore) in a Trans-blot SD semi-dry transfer cell (BioRad) for 1 hour at 300 mA per gel, limiting to 20 V, in SDS running buffer. The membrane was blocked with 5% Marvel milk powder (Premier Brands) in PBS for 1 hour and incubated with primary antibody diluted in PBS/5% milk powder. After 4 washes with PBS/0.1% Tween 20 (Sigma) the membrane was incubated with Protein-A-HRP (BioRad) diluted 1:1,000 in PBS/5% milk powder for

one hour. Following 4 washes with PBS/0.1% Tween 20 the reactive protein bands were visualized using the ECL chemiluminescence kit (Amersham Pharmacia), with blots being exposed to Kodak MXB X-ray film. The following dilutions of primary antibodies were used for western blotting

Rabbit 1064 α-X31 BHA	1:5,000
(provided by the World Influenza Centre (WIC), NIMR, London)	
Mouse α-influenza M1 monoclonal antibody (Serotec)	1:5,000
Mouse α-NP	1:1,000
(kindly provided by R. Webster, St. Jude Children's Research Hospital, Memphis, USA)	

2.2.2.3 Passaging of Cells

MDCK cells were cultured in 80 CM^2 flasks or 35 mm dishes (Nunc) in complete media at 37°C and 5% CO_2. All cells were adherent and were detached from the flasks with trypsin versene for passaging when confluent.

2.2.2.4 Freezing and Thawing of Cells

Confluent cells in a 175 CM^2 flask (Nunc) were trypsinised, diluted to 10 ml with DMEM and spun for 5 min at 500 g in a Beckman GS-6R centrifuge. The pellet was resuspended in 5 ml freezing medium consisting of 58.5% DMEM, 7.5% DMSO (Sigma) and 33% FCS. Aliquots of 0.5 ml were frozen at - 80°C in a polystyrene rack before transfer to liquid nitrogen. Cells were thawed quickly in a waterbath at 37°C, washed in 10 ml DMEM and resuspended in 1 ml complete medium. The cells were then transferred to a 80 CM^2 flask containing 30 ml complete medium and incubated at 37°C, 5% CO_2.

2.2.3 Virological Techniques

All continuous sucrose gradients for purification of virus or HA were prepared using a two-chamber gradient maker (prepared by NIMR engineering services) and a Minipulse 2 peristaltic pump (Gilson). Upon centrifugation fractions were taken using the Minipulse 2 peristaltic pump.

2.2.3.1 Source of Viruses

All virus seed stocks were provided by WIC (NIMR, London) either as allantoic fluid or as freeze-dried virus, except for Dk/Hokkaido/33/80, which was kindly provided by H. Kida (Hokkaido University, Japan).

2.2.3.2 Virus Growth in Eggs

Influenza viruses were grown in the allantoic cavity of 10-day-old embryonated hen's eggs. One hundred µl of virus (in dilutions ranging from 10^{-1} to 10^{-5}) were injected into each egg and the site of inoculation was sealed with paraffin wax. The eggs were incubated at 33°C for 48-72 hours and then at 4°C for 8-24 hours. The allantoic fluid was harvested, filtered through 0.45 µm filters (Sartorius) and kept at –80°C. Alternatively, virus was purified by sucrose gradient centrifugation as a modification of Skehel and Schild (1971): the allantoic fluid was clarified by centrifugation in a Beckman J6-MC centrifuge (900 g, 20 min, 4°C), followed by pelleting the virus in a Beckman Avanti J-25 Centrifuge (7,000 g, 12-15 hours, 4°C). Then the virus was resuspended in 10 mM Tris/150 mM NaCl pH 8 using a homogeniser (Kontes Glass Co.) with a loose pestle and subsequently purified by centrifugation through a 15-40% (w/v) continuous sucrose gradient in 10 mM Tris/150mM NaCl pH 8 in a BeckmanXL-90 Ultracentrifuge (85,000 g, 45 min 4°C). The visible white virus-containing band was removed, diluted to 30 ml with 10 mM Tris/150 mM NaCl pH 8

and pelleted by spinning in a Beckman XL-90 Ultracentrifuge (85,000 g, 90 min, 4°C). The pellet was resuspended in 1-3 ml HBS-EP buffer and stored at 4 °C. Virus titres were determined by haemagglutination or plaque assays.

2.2.3.3 Virus Growth in MDCK Cells

MDCK cells in 80 CM^2 flasks or 35 mm dishes were washed twice with warm DMEM medium. Then, 0.5 ml of virus (in dilutions ranging from 10^{-1} to 10^{-5}) was added and the virus left to adsorb for 30-45 min at room temperature. After removal of unbound virus by aspiration, complete medium supplemented with 2.5 μg/ml TPCK-treated trypsin (Sigma) was added (20 ml and 2 ml for flasks and dishes, respectively). Upon incubation at 37°C, 5% CO_2 for 48-72 hours, the cell supernatant was centrifuged at 500 g in a Beckman GS-6R centrifuge to remove cell debris and kept at - 80°C. Virus titre was determined by haemagglutination or plaque assays.

2.2.3.4 Plaque Assay

MDCK cells were seeded in 35 mm dishes and incubated at 37°C, 5% CO_2 until they were tightly packed. The cells were washed twice with warm DMEM and incubated with 500 μl 10-fold virus dilutions for 30 min at room temperature. Any unbound virus was removed by aspiration and 2 ml of overlay medium were added to each dish. After the overlay had set, the dishes were incubated at 37°C, 5% CO_2 until plaques became visible (2-4 days). Then the cells were fixed in 0.25% glutaraldehyde in PBS for 30 min at room temperature. The agar overlay was removed under running water and the cells were stained with 1% crystal violet (Sigma) in 20% ethanol. The cells were then washed with water to remove any excess dye to visualise the plaques. Alternatively, the plaques were picked with a glass

pasteur pipette (Volac) and resuspended in 500 µl DMEM for direct sequencing or growth in MDCK cells.

2.2.3.5 Haemagglutination Assay

Viral titres were determined in V-bottom microtitre plates (Greiner Labortechnik). Two-fold serial dilutions of 50 µl virus suspension in PBS were done and 50 µl of 1% (v/v) turkey RBC suspension in PBS were added to each well. The plates were read after incubation for 1 hour at room temperature and the virus titre (HAUs) expressed as the reciprocal of the maximum virus dilution that caused complete agglutination of RBC.

2.2.3.6 Haemagglutination Inhibition (HI) Test

Two-fold serial dilutions of antibody in 50 µl PBS were done in V-bottom microtitre plates. Then 8 HAUs of virus in 50 µl of PBS were added to each well and the plate was incubated for 30 min at room temperature. Fifty µl of a 1% (v/v) turkey RBC suspension in PBS were added to each well and the plate incubated at room temperature for a further 60 min to allow agglutination. The efficacy of inhibition by antibody or horse serum was determined as the reciprocal of the maximum dilution that inhibited agglutination of RBC.

2.2.4 Preparation of Bromelain-Released HA (BHA)

The preparation of BHA is based on the Method described by Compans et al.(1970). Virus at a final total protein concentration of 2-5 mg/ml was incubated with bromelain in a ratio of 2:1 (w/w) in 10 mM Tris/150 mM NaCl/50 mM mercaptoethanol/0.1% (w/v) sodium azide pH 8 at 37°C for 90 min. Then the viral cores were pelleted in a Beckman Optima Ultracentrifuge (150,000 g, 20 min, 4°C), and the supernatant was spun through a continuous 5-25% (w/v) sucrose gradient in 10 mM Tris pH 8 in a Beckman XL-90

Ultracentrifuge (180,000 g, 16 hours, 4°C). The remaining virus pellet was resuspended in 10 mM Tris/150 mM NaCl pH 8 and the bromelain digestion procedure was repeated for 4 hours and subsequently overnight. One ml fractions of the sucrose gradients were taken and BHA and NA detected by spectrophotometry and by SDS-PAGE. The majority of NA was usually removed in the 90 min digest, whereas HA was cleaved off the virus in subsequent digests. The gradient fractions containing BHA were pooled and the sucrose depleted by buffer exchange in 10,000 MWCO (molecular weight cut-off) Vivaspin 20 (Sartorius) into 10 mM Tris pH 8 (3 times 1:10 dilution, 1,200 g in a Beckman GS-6R Centrifuge). Further purification was achieved by ion-exchange using a Q15 (cation)-syringe filter (Sartorius). After equilibration with 10 ml 10 mM Tris pH 8, the sample was bound to the filter. Bound proteins were eluted with 2.5 ml 10 mM Tris pH 8 with increasing NaCl concentrations (0, 50, 100, 150, 200, 400 and 1,000 mM). Elution of BHA usually occurred at 150-400 mM NaCl, whereas any remaining NA was eluted at 50-100 mM NaCl, as determined by SDS-PAGE. The fractions containing BHA were pooled and any remaining NA was removed from the sample by affinity chromatography using a α-NA immunoaffinity column.

2.2.5 Preparation of α-NA Immunoaffinity Column

A 1 ml Hi-Trap rProtein A FF column (Amersham Pharmacia) was connected to a Minipulse 2 peristaltic pump and equilibrated with 20 ml PBS. X31 monoclonal α-NA antibody solution prepared by the method of Koehler and Milstein (1975) was diluted 1:3 with PBS and 20 ml pumped through the column for 10 min. Any unbound antibodies were washed off with PBS (detected by spectrophotometry). Upon equilibration with 10 ml 0.2 M triethanolamine pH 8.3, the antibody was crosslinked to the column with 20 ml 0.2 M triethanolamine/30 mM dimethyl pimalidate (Perbio) pH 8.3 for 45 min.

Subsequently, the reaction was stopped with 20 ml 0.3 M glycine pH 8.3 and any non-crosslinked antibody was removed with 5 ml 0.15 M citrate buffer pH 3. The column was washed with 20 ml PBS/0.1% (w/v) sodium azide and stored at 4°C.

2.2.6 Preparation of low-pH-induced BHA Rosettes

BHA at 1 mg/ml was adjusted to pH 5 with citrate buffer pH 3.5. At this pH, the BHA trimers undergo a conformational change associated with membrane fusion and form soluble aggregates called rosettes. Each particle contains about 6 to 10 trimers as estimated by electron microscopy and display multivalent binding characteristics of a virus, e.g. agglutination of RBC (Skehel et al., 1982). Upon incubation at room temperature for 10 min, the pH was adjusted to 7-8 with 1 M Tris pH 8. The rosettes were then separated from non-aggregated trimers by centrifugation through a continuous 5-25% (w/v) sucrose gradient with a 60% (w/v) sucrose cushion in 10 mM Tris pH 8 in a Beckman XL-90 Ultracentrifuge (180,000 g, 16 hours, 4°C). The rosette-containing fractions as determined by spectrophotometry and SDS-PAGE were pooled and then dialysed in 10,000 MWCO Slide-A-Lyzer cassettes (Perbio) against 4 L PBS for 48 hours at 4°C with one buffer change to deplete sucrose. For analysis using the BIAcore, PBS was exchanged for HBS-EP in 10,000 MWCO Vivaspin 20 (3 times 1:10 dilution, 1200 g in a Beckman GS-6R Centrifuge).

2.2.7 Neuraminidase Activity Assay

This assay is a modification of Warren (1959). Two-fold serial dilutions of virus or sample to be tested for NA activity were done in 50 µl HBS-EP buffer in 50 x 10 mm test tubes (SAMCO). A negative control containing only buffer was included. Fifty µl of complete fetuin substrate (diluted 1:3 in buffer) was added to each tube and the reaction was

incubated at 37°C for 15 hours. Then 50 µl periodate reagent was added to the reaction mixture and the tubes incubated at room temperature for 20 min. Arsenite solution (250 µl) was added and mixed until a yellow-brown colour disappeared. Then 500 µl thiobarbituric acid was added and the tubes incubated in a boiling water bath for 10 min. NA activity was detected by formation of a red colour and expressed as the reciprocal of the maximum virus or sample dilution yielding the colour formation.

2.2.8 Neuraminidase Activity Inhibition (NAI) Test

Two-fold serial dilutions of NA inhibitor were done in 50 µl HBS-EP buffer in 50 x 10 mm test tubes (SAMCO). Then 50 µl of virus sample was added and the reaction incubated for 30 min at room temperature. Upon addition of 50 µl complete fetuin substrate (diluted 1:3 in buffer) and incubation at 37°C for 15 hours, the NA activity was detected as described above and the minimal inhibitory concentration determined as the reciprocal of the maximum inhibitor dilution preventing the colour formation. The NA activity of the virus sample in absence of inhibitor was determined in parallel as a positive control.

2.2.9 Biotinylation of Fetuin and Asialofetuin

Fetuin and asialofetuin (Sigma) were biotinylated using the Immunoprobe biotinylation kit (Sigma). Biotinamidohexanoic acid-Sulfo-N-hydroxysuccinimide was mixed with fetuin or asialofetuin at a molar ratio of 5:1 in sodium phosphate buffer pH 7.2, and the reaction was incubated at room temperature for 30 min with gentle stirring. Any unincorporated biotin was removed by buffer exchange in 10,000 MWCO Vivaspin 20 (3 times 1:10 dilution, 1,200 g in a Beckman GS-6R Centrifuge) into PBS or 25 mM 2-morpholinoethanesulfonic acid (MES) (Sigma)/150 mM NaCl/2.5 mM $MgCl_2$ pH 6. The biotin/protein ratio was determined using the supplied avidin-2-hydroxyazobenzen-4'-

carboxylic acid assay using the extinction coefficient as described by the manufacturer.

2.2.10 Treatment of Turkey RBC with Viral Neuraminidases

Turkey RBC were treated with viral NA (X31 or Japan/305/57 (JAP)), prepared by bromelain digest and purification as described for production of BHA) in order to remove sialic mainly in the α(2,3)-linkage. One ml 10% (v/v) turkey RBC in PBS was spun down at 1,500 g in an Eppendorf 5415 C centrifuge and washed twice to yield a 10% (v/v) suspension in desialylation buffer. Indicated amounts (see *Chapter* 5) of sialidases were incubated with the blood at 37°C for 15-45 min. Then the blood was washed four times by pelleting the cells at 1,500 g in an Eppendorf 5415 C centrifuge and resuspension in 1 ml HEPES buffer. Removal of α(2,3)-linked sialic acid was assessed by haemagglutination assays with X31, Leu226Gln and Dk/Hokkaido/33/80 viruses or by binding of these viruses labelled with ^{125}Iodine to the treated turkey RBC.

2.2.11 De- and Resialylation of Turkey RBC

This assay is a modification of Carroll et al. (1981). One ml 10% (v/v) turkey RBC in PBS was spun down at 1,500 g in an Eppendorf 5415 C centrifuge and washed twice to yield a 10% (v/v) suspension in desialylation buffer. Then the blood cells were desialylated by incubation with 100 mU *Vibrio cholerae* NA (Sigma) at 37°C for 2 hours, followed by 5 washes with 1 ml HEPES buffer. The red cells were then reconstituted with sialic acid either in the α(2,6)- or the α(2,3)-linkage to galactose by use of linkage-specific sialyltransferases (α(2,3)-(N)-sialyltransferase, α(2,6)-(N)-sialyltransferase, Calbiochem): 200 µl 10% (v/v) desialylated red cells were incubated with 50 µl 15.3 mM cytidine-5'-monophospho-N-acetylneuraminic acid (CMP-Neu5Ac) (Sigma) and varying concentrations of transferases in a total of 1 ml resialylation buffer at 37°C for 4 hours.

The blood was then washed 3 times in 1 ml resialylation buffer and stored at 4°C. The binding capacity of X31, Leu226Gln and Dk/Hokkaido/33/80 viruses for the de- and resialylated blood was tested as described below and expressed as percentage compared to binding of untreated blood.

2.2.12 Selection Experiments

Two hundred and fifty µl of Dk/Ukraine/1/63 and Dk/Hokkaido/33/80 viruses at titres of 64-256 HAUs were added to 250 µl of resialylated turkey RBC at 1-0.001% (v/v) final concentrations. Viruses were allowed to bind to the cells for 10 min at room temperature. Unbound virus was removed by washing the blood 6 times in 500 µl HEPES buffer in an Eppendorf 5415 C centrifuge (1,500 g, 1 min). After every wash step, the resuspended blood was transferred to a new Eppendorf tube. Then the blood was resuspended in 1 ml desialylation buffer pH 6.5 and the virus was eluted from the cells by incubation with 15 mU *Vibrio cholerae* NA (Sigma) at 37°C for 1 hour. The RBC were then pelleted, the supernatant filtered through 0.45 µm filters and used for infection of MDCK cells in 6-well plates. Virus grown after selection with the lowest red cell concentration and with the 1% (v/v) red cell suspension was used for further selection assays. In parallel, Dk/Ukraine/1/63 and Dk/Hokkaido/33/80 viruses were passaged in MDCK cells to assess any mutations due to adaptation to growth in tissue culture cells.

2.2.13 Labelling of Virus with ^{125}Iodine (Bolton-Hunter Reagent)

X31, Leu226Gln and Dk/Hokkaido/33/80 viruses were labelled with ^{125}Iodine using the Bolton Hunter Reagent (Amersham Pharmacia). Four point six MBq of Bolton-Hunter reagent were transferred to an Eppendorf tube and dried in a stream of nitrogen gas. Then 300 µl of egg-grown-purified virus (equivalent to 768,000 HAUs) in 0.1 M sodium borate

pH 8.5 were added to the dry Bolton-Hunter reagent, mixed gently and incubated on ice for 15 min. The reaction was stopped by adding 0.5 ml 0.2 M glycine/0.1 M sodium borate pH 8.5 and incubation on ice for 5 min. Virus was separated from unincorporated Bolton-Hunter label by gel filtration using a BioRad column (30 x 0.7 cm) packed with Sephadex G25 (Sigma) and equilibrated with PBS/0.25% gelatine (Sigma)/0.05% (w/v) sodium azide. Half ml fractions were collected and 5 µl of each fraction transferred to 3.5 ml 55 x 12 mm tubes (Sarstedt) containing 0.25 ml 1 M NaOH. Radioactivity was measured for 5 min using a LKB 1277 Automatic Gamma Counter (Wallac) and expressed as counts per minute (cpm). The samples containing at least 10% of the total radioactivity within the first 10 fractions were pooled and the virus titre determined by the haemagglutination assay.

2.2.14 Binding Experiments of ^{125}Iodine-labelled Virus to Turkey RBC

Labelled virus was diluted 1:1,000 in HEPES buffer, generating a stock solution of 7,000-12,000 cpm and a haemagglutination titre of 256 HAUs. Then, 250 µl were added to 250 µl of a 2% (v/v) turkey RBC solution. Virus was allowed to adsorb to the cells for 30 min on ice. Unbound virus was removed by washing the cells twice in 0.5 ml HEPES buffer. Subsequently the cells were lysed in 500 µl 1 M NaOH for 30 min at 37°C, transferred to 3.5 ml Sarstedt tubes and the radioactivity counted for 5 min in a LKB 1277 Automatic Gamma Counter. The amount of bound virus was calculated as percentage of cpm compared to the original virus stock solution. All experiments were done three times and in duplicates.

2.2.15 Quantitation of Virus Particles for SPR Experiments

Virus concentration of X31 was determined based on the amount of NP protein present in

the virus preparation. Viral proteins were separated by SDS-PAGE in non-reducing loading buffer and stained with Commassie Blue. The net intensity of the NP band was then measured on a Kodak Image station 440 CF with the Digital Science 1D software (Perkin Elmer), and the concentration calculated against a standard curve of BSA. The virus concentration was determined assuming a constant number of 590 copies of NP (MW = 56,000 Da) per influenza A virion. This number was calculated based on the influenza virus genome consisting of 13588 nucleotides and the binding of 23 nucleotides per NP monomer (Ruigrok, 1998). The relative amount of other viruses was calculated against a standard curve generated by X31 virus. Bovine serum albumin (BSA) or X31 virus was run at 5 different concentrations generating equally spaced datapoints for the generation of standard curves, whereas virus preparations for which the NP concentration was determined were run at two different dilutions on the same SDS-Gel. All the samples were run in duplicates.

2.2.16 Preparation of α(2,3)- and α(2,6)-Fetuin as Substrates for Binding Experiments

Fetuin containing Neu5Ac either in the α(2,6)- or α(2,3)-linkage to galactose was prepared by sialylation of biotinylated asialofetuin with CMP-Neu5Ac (Sigma) and linkage-specific transferase, α(2,6)-(N)-sialyltransferase and α(2,3)-(N)-sialyltransferase (Calbiochem), respectively. One hundred µl asialofetuin (at 0.95 mg/ml) in 25 mM MES (Sigma)/150 mM NaCl/2.5 mM $MgCl_2$ pH 6 were mixed with 1.8 mU α(2,3)-(N)-sialyltransferase or 0.6 mU α(2,6)-(N)-sialyltransferase and 50 µl CMP-Neu5Ac at concentrations described in *Chapter 3*, and sialylation was performed for 4 hours at 37°C. Unincorporated Neu5Ac was removed by buffer exchange in 10,000 MWCO Vivaspin 20 into PBS (4 times 1:10 dilution, 1,200 g in a Beckman GS-6R Centrifuge). In order to monitor incorporation of unlabeled CMP-Neu5Ac, a trace amount of CMP-[^{14}C]-Neu5Ac (325.2 mCi/mmol; Perkin

Elmer) was added to give a final specific activity of 93-373 cpm/nmol. After buffer exchange, the concentration of sialylated fetuin in 1 ml PBS was determined by spectrophotometry. Then 650 µl were transferred into 20 ml scintillation vials (Packard) containing 9 ml Ready Safe liquid scintillation cocktail (Beckman) and the radioactivity as cpm measured by counting for 1 min in a LS 6000IC Scintillation Counter (Beckman) with colour quench correction set with limits of 4.567 to 309.63.

2.2.17 Surface Plasmon Resonance Binding Experiments

Interactions between HA-rosettes or virus (analyte) and fetuin (ligand) were measured on a BIAcore 2000 biosensor system (BIAcore AB, Uppsala, Sweden) at 25°C. Fetuin and asialofetuin (Sigma) were biotinylated as described above, diluted to 50 µg/ml in PBS, filtered through 0.22 µm filters (Sartorius) and stored at –20°C. Alternatively, biotinylated asialofetuin was restored with Neu5Ac in the α(2,3)- or α(2,6)-linkage as described and stored like fetuin at 50 µg/ml. Then they were immobilized onto streptavidin sensor chips by injection over the sensor surface of different flow channels at a constant flow rate of 5 µl/min for 7 min. Non-specifically bound protein was removed by 3 consecutive injections of 5 µl 10 mM glycine/0.5% Tween 20/NaOH pH 11.5 (regeneration solution). Analyte-ligand interaction was analysed using HBS-EP (BIAcore AB) as running buffer. Analyte was injected over the sensor surface at a flow rate of 10 µl/min using the Kinject command. After every binding experiment, regeneration solution was injected consecutively for 1 min over the sensor surface until all bound analyte was removed from fetuin. The results were analysed using standard non-linear least square fit methods and the kinetic parameters calculated as described in *Chapter 3*.

3 Development of a Receptor-Binding Assay for Influenza Virus and Receptor Analogues Based on Surface Plasmon Resonance

3.1 Introduction

3.1.1 Available Receptor-Binding Assays

A number of different assays have been developed for the study of influenza virus receptor-binding activity. The earliest available methods were virus-induced haemagglutination assays using RBC from different animal species (e.g. human, chicken, guinea pig, horse) (Hoyle, 1968a; Ito et al., 1997a) or enzymatically modified erythrocytes. These modifications include potassium periodate oxidation, leading to removal of the sialic acid glycerol chain (Underwood, 1985), and reconstitution of sialic acid-depleted RBC either with sialic acid species in different linkages (Paulson et al., 1979; Higa et al., 1985; Paulson and Rogers, 1987) or with incorporated gangliosides (Suzuki et al., 1985; Suzuki et al., 1986). While these assays have proven to be useful in detecting relative differences in recognition of these erythrocytes, the complex nature of the agglutination reaction and the lack of detailed information on the structure, composition and density of red cell glycoproteins and glycolipids do not allow a quantitative analysis of the interaction being studied. Although adsorption of virus to RBC can be measured in order to overcome the complexity of the agglutination reaction (Rogers and Paulson, 1983), the limitations mentioned above regarding the substrates used still apply. Furthermore, reproducibility of results is limited by the instability of RBC. Other assays providing relative information on receptor-binding properties involve binding of virus to gangliosides on thin layer chromatography (TLC) plates (Suzuki et al., 1992) and the interaction of HA-expressing cells with erythrocytes loaded with horseradish peroxidase (HRP) (Martin et al., 1998).

In contrast to the qualitative nature of the described assays, quantitative receptor-binding information can be obtained by nuclear magnetic resonance (NMR) studies. The affinity of the interaction between HA trimers or virus and soluble monovalent receptor analogues is expressed by the equilibrium dissociation constant (K_D), which has been determined to be approximately 10^{-3} M (Sauter et al., 1989; Hanson et al., 1992). Millimolar dissociation constants have also been determined for other viruses recognising sialic acid, e.g. polyomavirus and rhesus rotavirus (Stehle and Harrison, 1996; Dormitzer et al., 2002). Studies with lectins have confirmed this extremely weak carbohydrate-protein interaction (Weis and Drickamer, 1996), which is in contrast to typical high-affinity protein-protein interactions of virus and receptor ($K_D = 10^{-6}$-10^{-9} M) (Lasky et al., 1987; McDermott et al., 2000). However, clustering of lectins and the abundance of HA trimers on the virus particle leads to the simultaneous binding of multiple receptors. This phenomenon is referred to as multivalency, which results in very tight binding of cell surface receptors. Although real equilibrium constants are obtained by NMR studies, the major drawbacks of this technique are the requirement for milligram quantities of protein and the extensive use of a high-field NMR spectrometer.

Competition with a substrate of known affinity for the receptor-binding site provides an alternative method of determining the binding strength of a ligand for HA or virus. The affinity can be expressed either as the substrate concentration needed to inhibit binding of another ligand by 50% (relative potency) or can be calculated directly. This approach has been used in fluorescence polarisation studies (Weinhold and Knowles, 1992) and adsorption of virus to RBC (Pritchett et al., 1987). Similarly, the relative potency for inhibition of haemagglutination can be determined (Toogood et al., 1991). A routinely used assay is the interaction of virions attached to a solid phase with soluble HRP-labelled sialylglycoproteins or competition of these with unlabelled ligands,

analogous to a sandwich ELISA (Gambaryan and Matrosovich, 1992). However, quantitative analysis by competition assays requires well-characterised ligands and is often complicated by differences in their valency.

3.1.2 Studies of Biomolecular Interactions by Surface Plasmon Resonance

All the quantitative assays described above determine the binding strength of an interaction by measuring the concentration of free and bound substrates at equilibrium, where no net change in complex concentration is observed. However, this approach is often limited by the length of time required for many biological reactions to reach equilibrium at reasonable substrate concentration and does not yield kinetic constants for the rates of association (k_a) and dissociation (k_d).

Biosensors permitting the study of interactions based on SPR were first introduced in 1990 and have developed rapidly since then (Fagerstam et al., 1992; Biacore, 1998). These permit real-time analysis of receptor-ligand binding specificity, affinity and kinetic constants, and can also provide information on the stoichiometry, cooperativity and thermodynamics of a reaction. This method enables the use of substrates with molecular weights from hundreds of daltons (Davis and Wilson, 2000) to whole-cell dimensions (Quinn et al., 2000), and the measurement of effective affinities from sub-picomolar to greater than millimolar (Myszka et al., 1998) can be measured over a wide range of chemical conditions. SPR is therefore considered a powerful tool for large-scale screening of binding events.

In BIAcore instruments (BIAcore AB, Uppsala, Sweden), molecular interaction is detected on a sensor chip, consisting of a dextran-coated gold surface (sensor surface) mounted on a glass side. One of the components of the interaction (ligand) is immobilised onto the sensor surface, forming one wall of a flow cell, through which the other

interactant (analyte) is injected (see Figure 15a). Binding of analyte to ligand is detected by SPR, an optical phenomenon arising from the interaction of light with a metal surface (Kretschmann and Raether, 1968). In binding experiments monochromatic polarised light is directed at the sensor surface at various incident angles under conditions of total internal reflection. However, since the glass is covered by gold, this reflection is not total but a fraction of the light is absorbed by mobile electrons in the metal film. At a particular incident angle of the light, a wave of excited electrons (the plasmon resonance) is produced at the gold layer, which leads to almost complete absorption of the light. This is detected as a marked reduction of intensity of reflected light. Since an evanescent wave associated with the electron oscillation travels a short distance from the gold film, the SPR angle is dependent on and proportional to the refractive index of the medium near the sensor surface. The interaction of analyte and ligand leads to an increase of mass, which is proportional to an increase in refractive index. Therefore, a change of the SPR angle reflects differences in mass at the sensor surface. The angle of the reduced-intensity reflected light is continuously recorded by a detector and expressed in resonance units (RUs). A SPR angle change of 0.1° corresponds to 1,000 RUs, which requires the binding of ~ 1 ng/mm^2 to the sensor surface for the majority of proteins (Stenberg et al., 1991). Plotting the changes of the resonance signal as a function of time before, during and after injection of substrate generates a sensorgram, which is depicted in Figure 15b. A liquid handling system ensures constant flow of buffer and controlled transport of samples to the sensor surface.

In virology, SPR is a widely used method to study receptor-ligand interactions with either soluble viral proteins or whole virus particles (e.g. Casasnovas and Springer, 1995; McDermott et al., 2000). For influenza virus, an SPR-based assay has been established to measure the affinity of HA for a receptor analogue (Takemoto et al., 1996). Since the

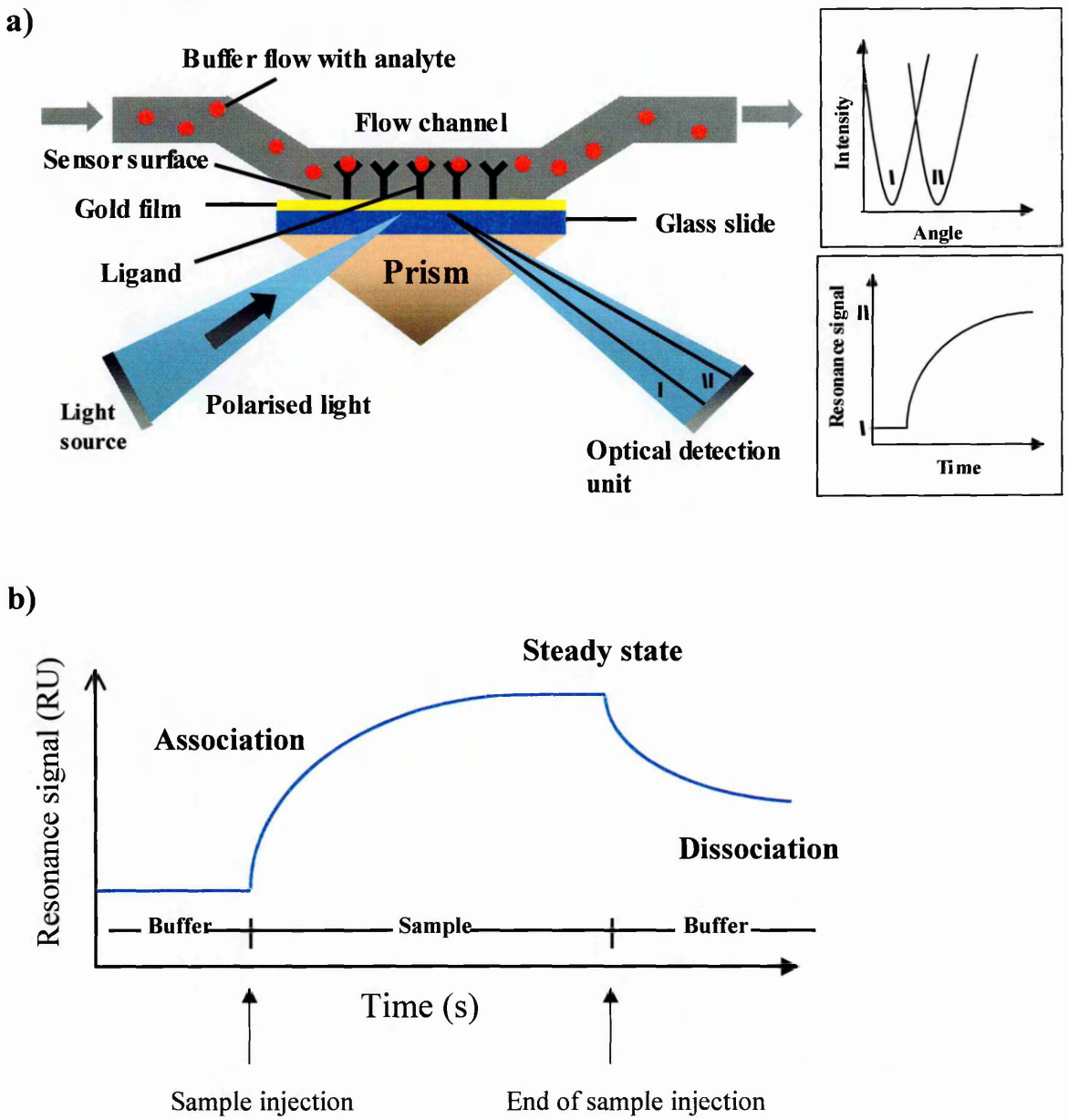


Figure 15 Principle of detection of molecular interactions by SPR

a) Components of a typical biosensor (adapted from Biacore, 2003a). A glass slide containing a thin gold film is mounted on a prism. Light at different incident angles is directed against the sensor surface onto which one interactant (ligand) is captured, reflects off the gold film and passes back through the prism to a detector. Samples containing the other interactant (analyte) are injected over the sensor surface. Interaction of analyte with ligand is detected as changes in the light angle at which SPR creates a large reduction in the intensity of reflected light. This change is proportional to the mass of bound analyte close to the sensor surface and is expressed in resonance units (RUs).

b) Monitoring changes in SPR angles as a function of time creates a sensorgram. Analyte binds to ligand during sample injection, resulting in an increase of signal. At steady-state, no more net complex formation is observed. At the end of the injection, the sample is replaced by continuous flow of buffer and the decrease in signal reflects dissociation of analyte from ligand.

interaction of HA with monovalent receptor analogues is of low affinity, as determined by NMR, the binding of low-pH rosettes (aggregates of BHA produced by treatment with low-pH) to fetuin, a sialylated glycoprotein from foetal calf plasma, was studied. The presence of multiple Neu5Ac moieties per fetuin molecule and BHA per rosette ensured multivalency, which enhanced the otherwise low affinity. Fetuin was immobilised on the sensor chip and the rosettes were injected over it.

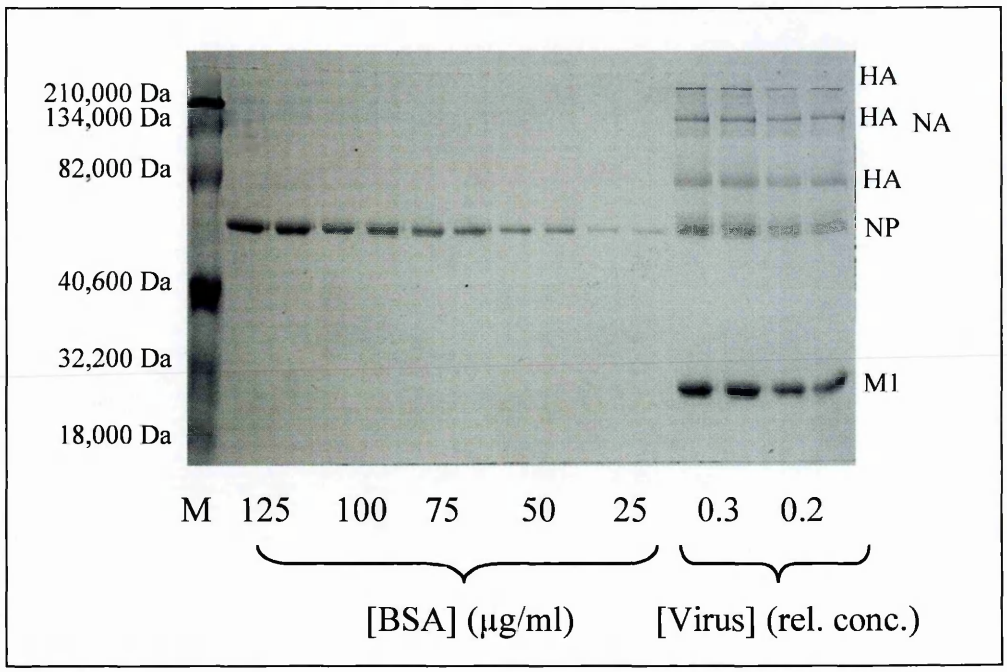
The aim of the following experiments was to investigate the possibility of using virus particles in affinity and specificity studies using SPR.

3.2 Virus-Receptor analogue Interaction Studies by SPR (BIAcore biosensor system)

3.2.1 Quantitation of Virus Particles

In order to gain quantitative information on kinetic and equilibrium constants, the concentrations of the reactants injected over the sensor surface need to be determined. Since binding of whole virus to fetuin was studied, quantitation of either HA or virus particles was required. However, the amount of HA in a virus preparation proved difficult to determine. Quantitation using extinction coefficients or colorimetric assays requires pure samples of HA produced by either bromelain digestion or disruption of virus with detergent followed by purification by sucrose gradient, and therefore does not provide accurate concentration values for the virus preparation. Therefore, viral proteins were separated by SDS-PAGE, stained with Coomassie Blue and their intensity compared to BSA standards (Perbio) ranging from 25-125 μg/ml (see Figure 16a). NP is difficult to separate from HA_1, as is M1 from HA_2, by SDS-PAGE due to their similarity in molecular weights. Therefore, the virus samples were run non-reduced, where HA runs as a single band. However, HA was partially retained in the stacking gel, as determined by Western

a)



b)

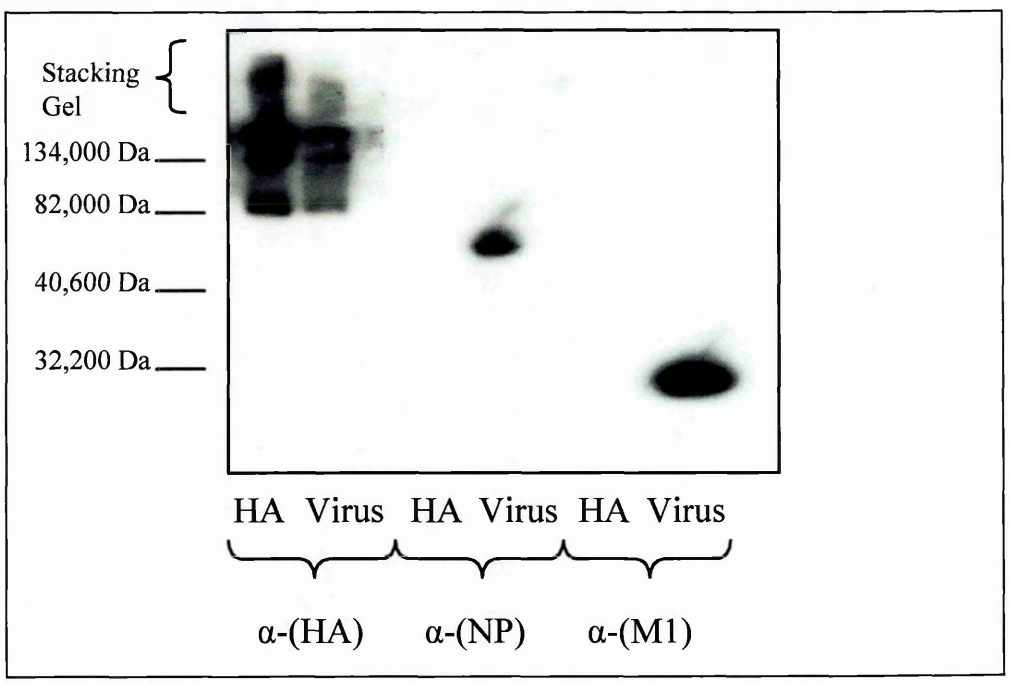


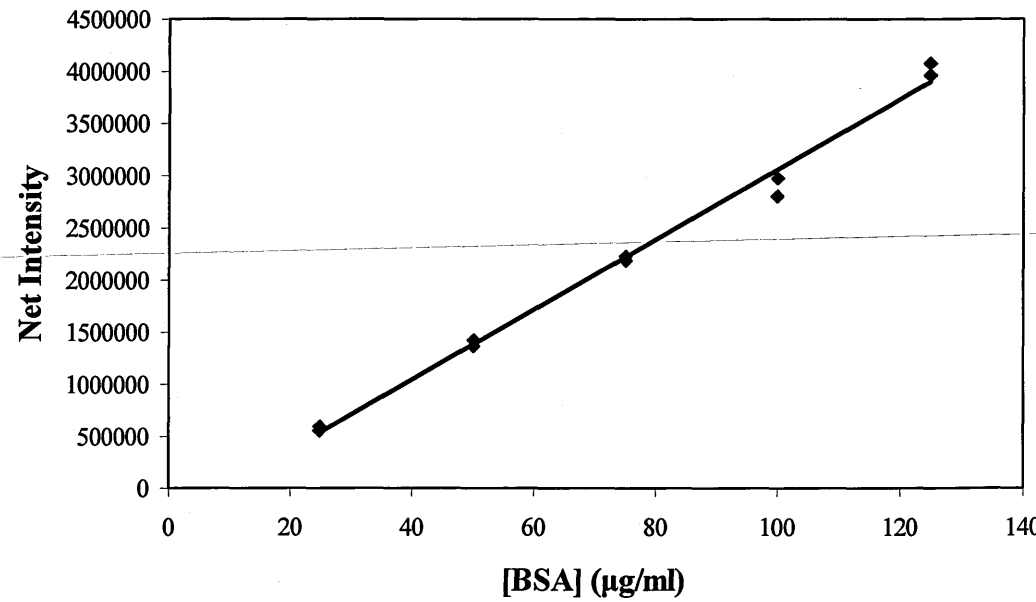
Figure 16 Quantitation of virus particles (1)
a) SDS-PAGE gel stained with Coomassie Blue showing the separation of viral proteins and BSA standards. Virus sample (X31) was run at two different concentrations compared to stock solution in duplicates. BSA standards were run at five different concentrations in duplicates in order to generate a standard curve (see Figure 17). All samples were run non-reduced for ease of separation of viral proteins. M = protein size marker **b)** Western blot of viral proteins showing that HA but not NP or M1 is partially retained in the stacking gel. Viral proteins were probed with α-(HA), α-(NP) and α-(M1)-antibodies. As a control for the specificities of the antibodies, full-length HA (prepared as described by Wharton et al. (1986)) was included in the experiment.

blotting (see Figure 16b), and this approach was therefore not considered to be appropriate for concentration determination. Furthermore, HA generated multiple bands of different sizes due to the presence of a mixture of monomers, dimers and trimers, complicating the analysis. Therefore, the concentration of virus particles was determined instead. Since the concentration of NP per virus particle is expected to vary the least due to coating of a constant number of 8 genome segments, this protein was quantitated by SDS-PAGE as described in *2.2.15 Quantitation of Virus Particles for SPR Experiments, p.88.* Unlike HA, this protein runs as a single band and is not retained in the stacking gel, as shown in Figure 16b. The BSA standard curve and the results of the quantitation are shown in Figure 17a and b. Virus particle concentration was determined by this method for X31 virus, which was in turn used to generate standard curves for the other viruses used in SPR studies. It is estimated that virus concentrations can be determined to within 5-15% using this approach. Errors of this magnitude would not affect the conclusions of the studies reported here. The total protein concentration of a 0.26 nM virus sample is estimated to be ~ 15 μg/ml, based on protein determination assays.

3.2.2 Bovine Fetuin as Receptor Analogue

The interaction of virus with Neu5Ac acid was initially studied using bovine fetuin as receptor analogue. This glycoprotein contains Neu5Ac in both the α(2,3)- and α(2,6)-linkage and was the first foetal protein to be described (Pederson, 1944). It contains six carbohydrate moieties/molecule, three N-linked to Asn (Spiro, 1962; Spiro, 1964) and three O-linked to Ser/Thr (Spiro and Bhoyroo, 1974). These account for 80% and 20% of total carbohydrate, respectively, and their structures have been extensively studied. Three distinct structures have been identified for the O-linked oligosaccharides, which are shown in Figure 18a (Spiro and Bhoyroo, 1974; Edge and Spiro, 1987). In contrast, many

a)



b)

Virus concentration (relative to stock solution)	NP net intensity measured	calculated NP in stock solution (µg/ml)
0.3	3048493	332
0.3	3067836	333
0.2	1969925	337
0.2	1746325	303

mean NP concentration (µg/ml)	standard deviation
326	15.4 (4.7%)

Figure 17 Quantitation of virus particles (2)
a) BSA standard curve derived from SDS-PAGE gel shown in Figure 16. The net intensity of the BSA standards stained by Coomassie Blue was determined on a Kodak Image station 440 CF and plotted against BSA concentration. **b)** Quantitation of NP concentration. The BSA standard curve was used to calculate the NP concentration of the virus sample run on the same SDS-PAGE gel as the BSA standards. The determined concentration was then used for the quantitation of virus particles as described in *2.2.15 Quantitation of Virus Particles for SPR Experiments, p.88.*

a)

NeuAcα(2,3)Galβ(1,3)GalNAc -O

NeuAcα(2,3)Galβ(1,3)┐
GalNAc -O
NeuAcα(2,6)┘

NeuAcα(2,3)Galβ(1,3)┐
GalNAc-O
NeuAcα(2,3)Galβ(1,4)GlcNAcβ(1,6)┘

b)

R-Galβ(1,4)GlcNAcβ(1,2)Manα(1,6)┐
Manβ(1,4)GlcNAcβ(1,4)GlcNAc -N
Galβ(1,4)GlcNAcβ(1,2)Manα(1,3) ┘
R-Galβ(1,4)GlcNAcβ(1,4) ┘

R-Galβ(1,4)GlcNAcβ(1,2)Manα(1,6)┐
Manβ(1,4)GlcNAcβ(1,4)GlcNAc -N
NeuAcα(2,6)Galβ(1,4)GlcNAcβ(1,2)Manα(1,3)┘
NeuAcα(2,3)Galβ(1,4)GlcNAcβ(1,4)┘

R-Galβ(1,4)GlcNAcβ(1,2)Manα(1,6)┐
Manβ(1,4)GlcNAcβ(1,4)GlcNAc -N
NeuAcα(2,6)Galβ(1,4)GlcNAcβ(1,2)Manα(1,3)┘
NeuAcα(2,3)Galβ(1,3)GlcNAcβ(1,4)┘
NeuAcα(2,6)┘ **R = NeuAcα(2,6) or NeuAcα(2,3)**

Figure 18 Structures of the major oligosaccharides of bovine fetuin
a) Composition of the three oligosaccharides linked to Ser or Thr (O-linked), which make up 20% of total carbohydrate of fetuin **b)** Composition of the three major oligosaccharides linked to Asn (N-linked), which make up 80% of total carbohydrate of fetuin.

different structures have been identified for the N-linked carbohydrates, displaying heterogeneity in the number of peripheral branches, the extent of sialylation and the location and linkage of Neu5Ac (Green et al., 1988; Bendiak et al., 1989; Cumming et al., 1989). The major N-linked carbohydrate structures as determined by high-pH anion-exchange chromatography are shown in Figure 18b (Townsend et al., 1989). The molar ratio of α(2,6) to α(2,3) has been shown to be 31:19 for N-linked and 7:32 for O-linked oligosaccharides in fetuin (Edge and Spiro, 1987; Cointe et al., 1998). Taking into account the predominance of N-linked (80%) over O-linked (20%) oligosaccharides, fetuin appears to contain more α(2,6)- than α(2,3)-linked Neu5Ac. However, since the relative proportions of the major N-linked oligosaccharides vary significantly in bovine fetuin from different sources (Townsend et al., 1989), it is difficult to calculate the exact ratio of the two linkages.

3.2.3 Immobilisation of Fetuin onto Streptavidin-Coated (SA) Sensor Surface

Different methods are available for covalent immobilisation or capturing of proteins on the sensor surface. Of these, coupling to streptavidin (SA)-coated sensor chips by biotin was chosen. Therefore, fetuin and asialofetuin were biotinylated to a molar biotin to protein ratio of 1 to 1.5 as described in *2.2.9 Biotinylation of Fetuin and Asialofetuin, p.85.* The biotin supplied by the kit contained a 6-carbon linker, allowing for improvement of interaction between streptavidin and biotinylated macromolecules (Green et al., 1971). Biotinylated fetuin was then injected over the sensor surface of the SA chip as described in *2.2.17 Surface Plasmon Resonance Binding Experiments, p.90*, producing a stable signal of ~ 1,000 RUs, serving as the baseline for binding experiments (see Figure 19a). This signal of ~ 1,000 RUs corresponds to a fetuin density on the sensor surface of ~ 1 ng/mm^2 (Stenberg et al., 1991). Asialofetuin was immobilised on a different flow channel. Then

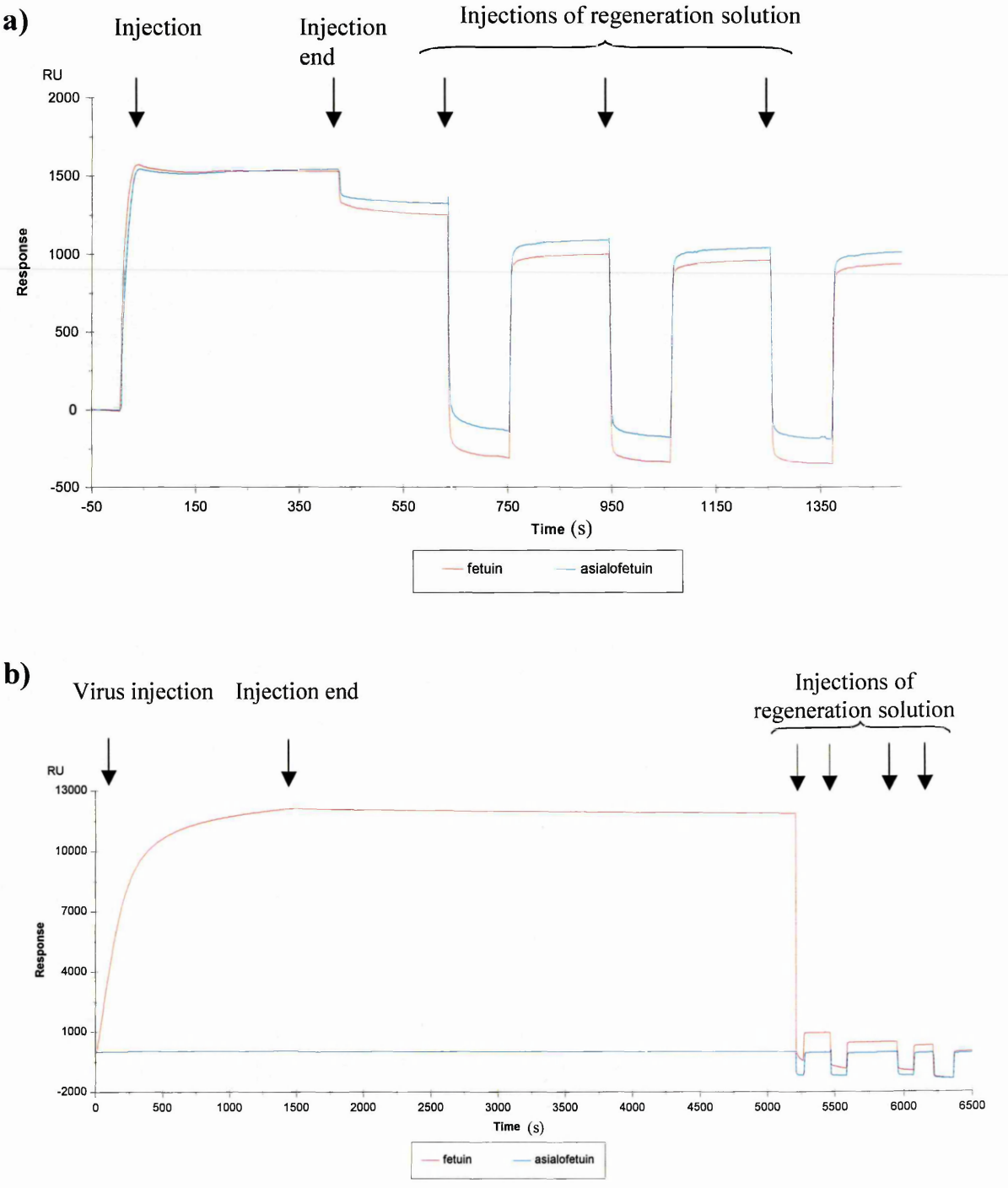


Figure 19 Interaction of X31 virus with fetuin and asialofetuin measured by SPR
a) Overlay of sensorgrams for binding of fetuin (red) and asialofetuin (blue) to the sensor surface of different flow channels. Upon sample injection, non-specifically bound protein was removed from the sensor surface by consecutive injections of regeneration solution. **b)** Association and dissociation curves for the interaction of X31 virus with fetuin (red) and asialofetuin (blue). Virus was injected at a concentration of 0.26 nM over the sensor surface for 25 min, followed by buffer flow for 45 min. The baseline was regenerated by injection of regeneration solution. No binding to asialofetuin was detected.

X31 virus was injected over the sensor surfaces containing either fetuin or asialofetuin. This laboratory-derived reassortant virus contains the HA and NA of the first H3 subtype virus isolated in 1968 (A/Aichi/2/68) and the remainder of the gene segments of an H1 subtype (Kilbourne, 1969). As can be seen in Figure 19b, the virus-fetuin interaction is solely mediated by Neu5Ac, since no binding to asialofetuin could be detected.

3.2.4 Virus-Fetuin Interaction Studies by SPR

The interaction of virus particles with immobilised fetuin was studied using X31 virus and its single-site mutant Gly225Asp. The HA of this virus has previously been shown to bind approximately 2-fold more weakly to RBC compared to X31 in a receptor-binding assay using HA-expressing cells (Martin et al., 1998). The Gly225Asp virus was therefore used to test the sensitivity of the SPR assay. The viruses were grown in eggs, purified (see *2.2.3.2 Virus Growth in Eggs, p.80*) and quantitated as described above. In order to prevent removal of Neu5Ac from immobilised fetuin, the virus sample and running buffer were supplemented with the NA inhibitor oseltamivir carboxylate, the active metabolite of oseltamivir (kindly provided by Roche). This inhibitor has been shown to be active across all nine influenza A NA subtypes, including the highly pathogenic H5N1 and H9N2 influenza viruses isolated from humans (Gubareva et al., 2000; Leneva et al., 2000). The final concentration of 10 μM is in large excess over an estimated requirement of 25 nM necessary for complete inhibition of the NA activity of X31 virus particles in the binding studies. This was determined by NAI studies as described in *2.2.8 Neuraminidase Activity Inhibition (NAI) Test, p.85,* with the virus control displaying NA activity of 1,280 units. In order to obtain reliable kinetic data, the viruses were injected over the sensor surface at different concentrations for 25 min. The highest concentration of virus particles used for SPR experiments was 0.26 nM, which corresponds to ~ 4 x 10^{10} virus particles in a volume

of 250 µl injected over the sensor surface. After every binding experiment, virus was removed from fetuin by consecutive injections of regeneration solution, resulting in restoration of the baseline. An overlay of the sensorgrams for X31 and Gly225Asp virus binding experiments is shown in Figure 20a and b. The kinetic constants were calculated as described below. Derivations of these equations are described in more detail in Appendix 2, p.255.

3.2.5 Calculation of Association Kinetics

In the simplest case, the signal in a sensorgram, at any given time during complex formation R(t), is described by the equation:

$$R(t) = \frac{k_a[V]R_{\max}(1-e^{-k_{obs}t)})}{k_a[V]+k_d} = A(1-e^{-k_{obs}t}) \qquad (1)$$

with $A = \frac{k_a[V]R_{\max}}{k_a[V]+k_d}$

R_{max} = the maximal response that would be obtained if all available binding sites were occupied, [V] = virus concentration, k_a = association rate constant, k_d = dissociation rate constant and k_{obs} = observed rate constant. A is the amplitude of the reaction curve and is also referred to as R_{eq}. This equation corresponds to a single exponential decay in which k_{obs} is equal to:

$$k_{obs} = k_a[V]+k_d$$

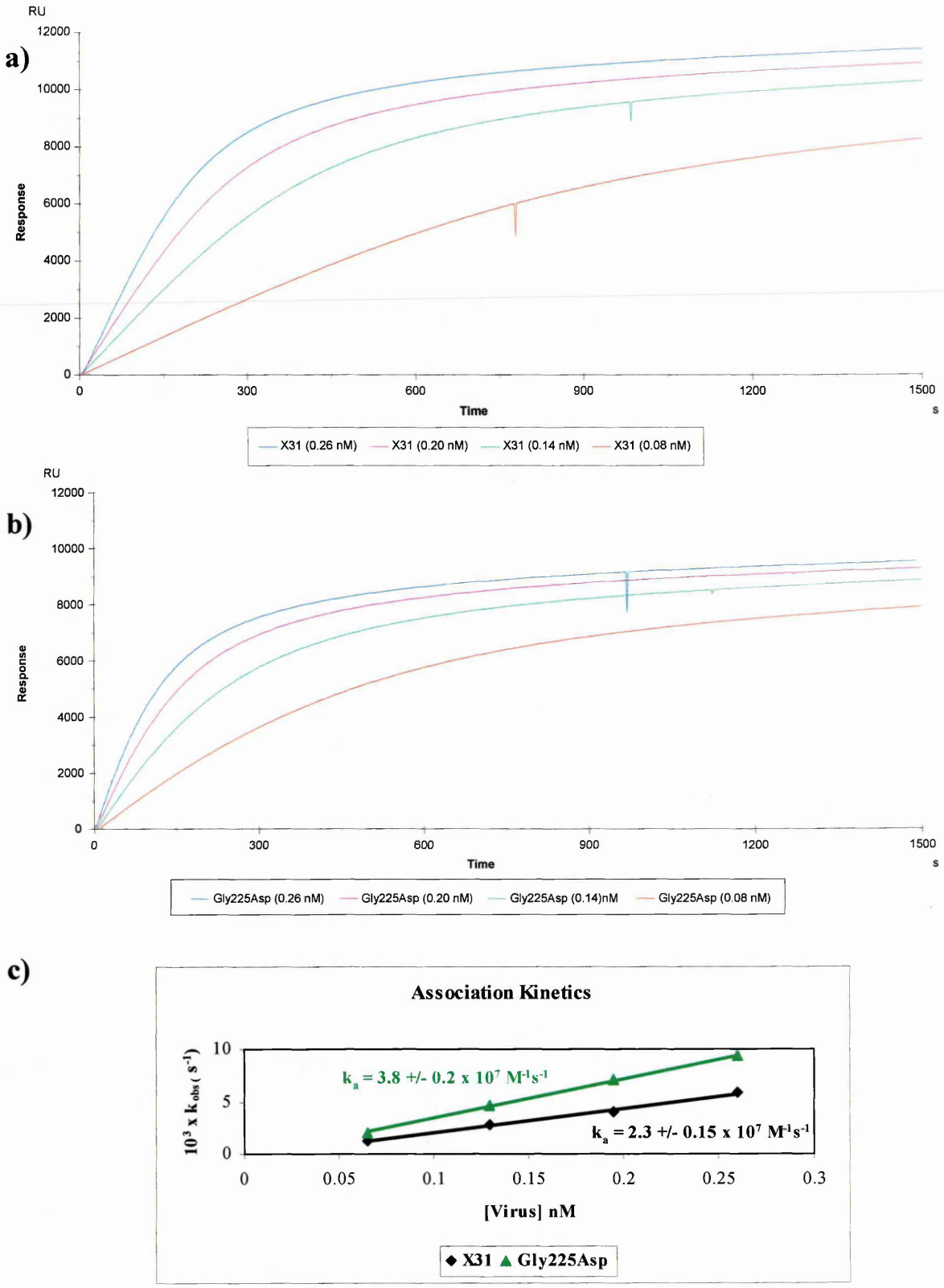


Figure 20 Calculation of association rate constants (k_a) for the interaction of X31 and Gly225Asp with fetuin X31 **(a)** and Gly225Asp **(b)** were injected over the sensor surface at four different concentrations for 25 min. The observed rate constant (k_{obs}) was determined for each of the association curves as described in *3.2.5 Calculation of Association Kinetics, p.104*. A plot of k_{obs} against virus concentration gives a straight line with slope k_a **(c).**

k_{obs} was calculated for sensorgrams generated at different virus concentrations by non-linear least squares regression analysis, and a plot of k_{obs} against the virus concentration gives a straight line with slope k_a. As seen in Figure 20c, Gly225Asp virus binds to fetuin about 2-fold faster than X31 virus. In theory, the intercept on the ordinate gives the value for k_d. In practice, however, measurements of the dissociation rate constant by this method are not reliable, since k_d is usually small in relation to $k_a[V]$, and slight errors of the slope have large effects on the measured intercept. The dissociation rate was therefore determined in separate experiments.

3.2.6 Calculation of Dissociation Kinetics

Dissociation of virus from fetuin is observed upon replacement of sample injection by buffer. Assuming negligible re-association of released virus (due to effective removal from the flow cell by the buffer flow), the decrease of signal can be described by the equation:

$$R(t) = R_0 e^{-k_d (t - t_0)} \qquad (2)$$

with R_0 = the response at an arbitrary starting time t_0 and k_d = dissociation rate constant. k_d is then calculated by non-linear least squares regression analysis.

Dissociation of virus from fetuin was observed for 45 min. The dissociation rate constant could be determined in a single experiment because it is concentration-independent. As shown in Figure 21, dissociation was very slow for both viruses but was significantly faster for the Gly225Asp virus. The affinity was then expressed as $K_D = k_d/k_a$. The rate constants and affinities are summarised in Table 1.

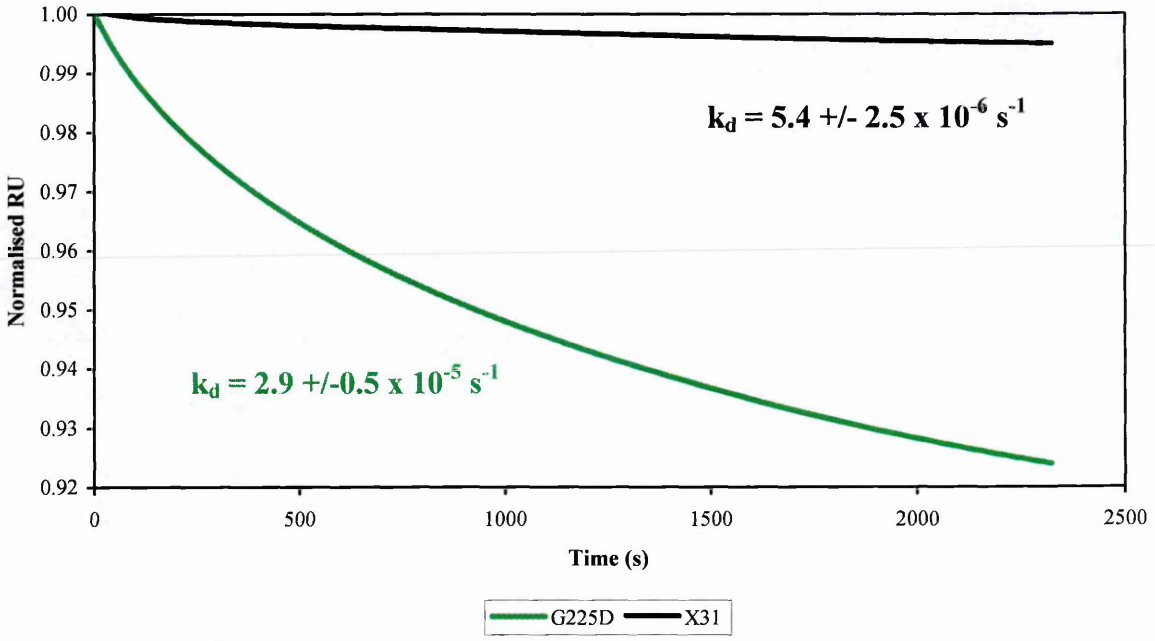


Figure 21 Calculation of dissociation rate constants (k_d) for the interaction of X31 and Gly225Asp with fetuin After virus injection at a concentration of 0.14 nM for 25 min, dissociation of virus from fetuin was observed for 45 min. The k_d was calculated from the single-exponential decay equation as described in *3.2.6 Calculation of Dissociation Kinetics, p.106.* The dissociation curves were normalised by setting the total reaction amplitude to 1. The total reaction amplitude is the signal at the point where buffer flow starts minus the baseline signal immediately prior to virus injection.

Virus	k_a ($M^{-1}s^{-1}$)	k_d (s^{-1})	K_D (M)
X31	2.3 +/- 0.15 x 10^7	5.4 +/- 2.5 x 10^{-6}	2.4 +/- 1.1 x 10^{-13}
Gly225Asp	3.8 +/- 0.2 x 10^7	2.9 +/- 0.5 x 10^{-5}	7.6 +/- 1.4 x 10^{-13}

Table 1 Summary of the kinetic rate constants and affinities for the interaction of X31 and Gly225Asp with fetuin The k_a and k_d were calculated from the association and dissociation curves shown in Figures 20 and 21 and the affinity expressed as $K_D = k_d / k_a$. The higher K_D value of Gly225Asp compared to X31 means that the virus' affinity for fetuin is lower than that of X31.

3.2.7 Reproducibility of Results

In order to establish the accuracy of the results and the stability of fetuin on the sensor chip, the association experiment was repeated with a different X31 virus batch. As can be seen in Figure 22, the calculated association rates are practically identical and deviations between the two experiments are negligible.

3.2.8 Deviations from Monophasic Association and Dissociation Kinetics

Simple homogeneous interaction kinetics are described by single exponential functions (see equations 1 and 2). However, non-linear regression analysis of the virus-fetuin interaction revealed that the association and dissociation curves were not in fact monophasic, particularly at the higher virus concentrations used (see Figure 23). The curves were therefore analysed using two-exponential functions,

$$R(t) = A_1(1 - e^{-k_{obs1}t}) + A_2(1 - e^{-k_{obs2}t}).$$

However, the majority of the total amplitude $(= A_1 + A_2)$ was associated with just one of the observed rates, which was used to calculate the kinetic rate constants. In the case of the association kinetics the k_{obs} for this major component varied linearly with virus concentration, confirming the validity of this approach.

3.2.9 Mass Transport Limitations

Before interaction with immobilised ligand can occur, the analyte in the flow cell needs to be transported from bulk solution to the surface of the sensor chip. This is described by the rate of mass transport (k_m), and the equation of complex formation is therefore written as:

$$A_{bulk} \underset{k_m}{\overset{k_m}{\rightleftharpoons}} A_{surface} + B \underset{k_d}{\overset{k_a}{\rightleftharpoons}} AB$$

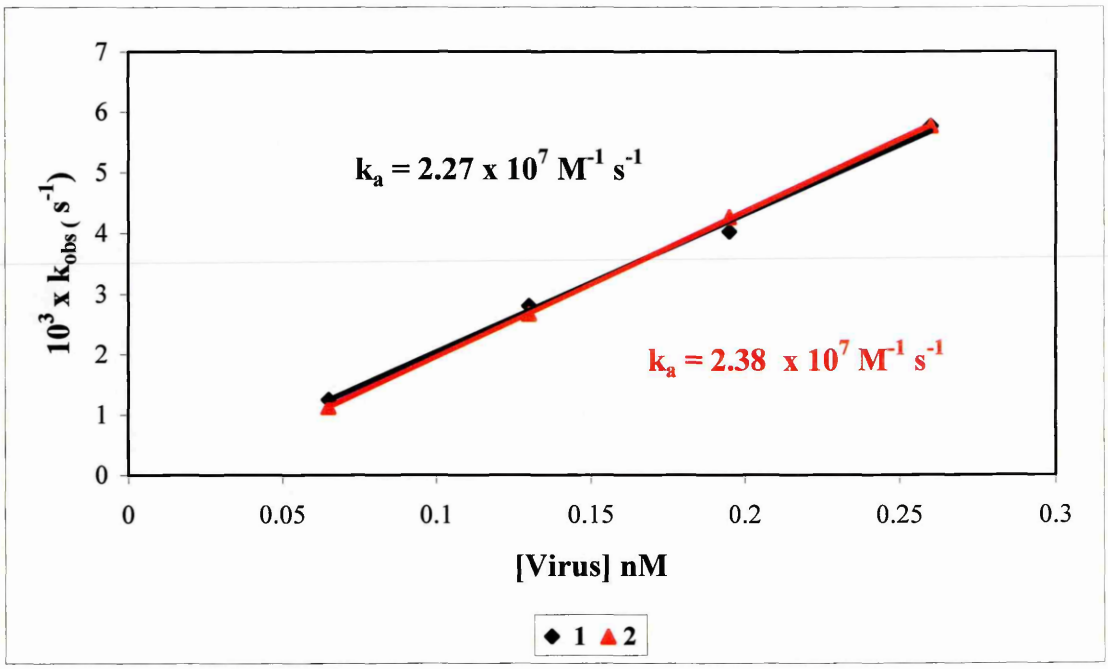


Figure 22 Reproducibility of SPR results

The association rate constant (k_a) was calculated for the virus-fetuin interaction using two different batches of X31 virus. k_{obs} was determined from association curves with four different concentration of virus as described in *3.2.5 Calculation of Association Kinetics, p.104*. Plots of k_{obs} against virus concentrations are shown in black and in red for the two different batches of virus, with the slope of this curve = k_a.

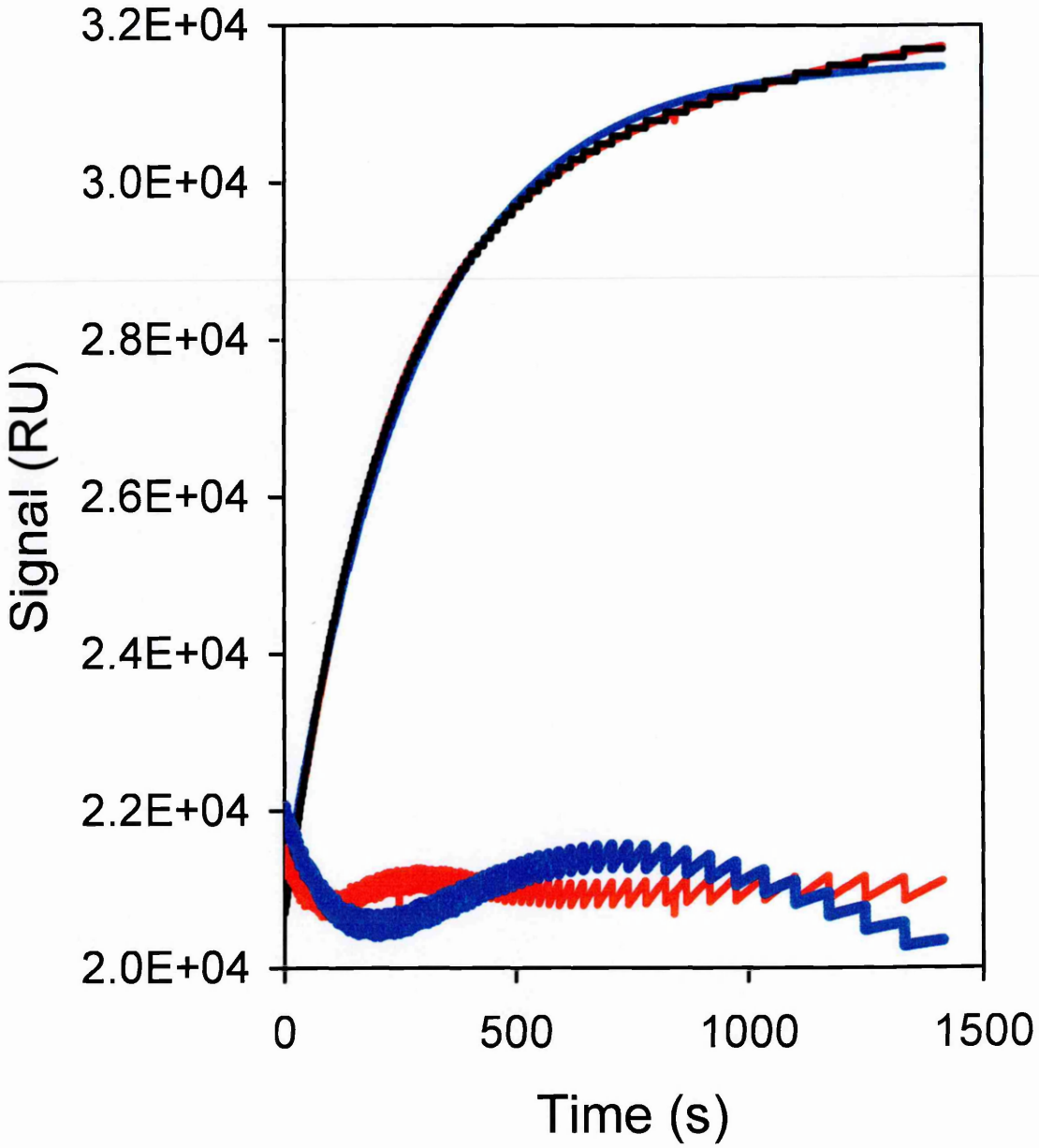


Figure 23 Fit of experimental association curve to a single- and a two-exponential curve A typical sensorgram for the interaction of virus with fetuin (black) was analysed using one- (blue curve) and two- (red curve) exponential functions. The residuals (multiplied by 3 and offset from zero) are indicated at the bottom and show that the single experimental curve is inadequate. The interaction is shown for X31 virus.

with analyte A, ligand B and k_m = rate for mass transport to and from the surface (the rate is the same in both directions). Mass transport is affected by flow cell dimensions and flow rate and also depends on the diffusion properties of the analyte.

The equations described above assume that the observed rate of binding reflects the interaction kinetics between analyte and ligand. This is the case if the rate of mass transport is much faster than k_a and k_d. If, however, this rate is considerably slower than the kinetic rates, the reaction is limited by mass transport, introducing artefacts predominantly at the beginning of association and the end of dissociation curves. An increase in flow rate and reduction of surface binding capacity (low concentration of immobilised ligand) reduce these limitations. In order to assess whether the heterogeneous kinetics are a result of mass transport limitations, the initial association rate constant was measured at different flow rates. A substantial increase (> 15%) of this rate serves as an indication for this effect. However, as seen in Figure 24, the virus-fetuin interaction is not markedly limited by mass transport.

3.2.10 BHA-Rosettes-Fetuin Interaction Studies by SPR

In order to investigate the effect of number of HA molecules per particle and particle size on the interaction kinetics, SPR experiments were also performed with BHA-rosettes prepared from X31 virus. Rosettes have been shown to contain an average of 6-10 BHA trimers and display multivalent binding characteristics based on their ability to agglutinate RBC (Skehel et al., 1982).

Rosettes were prepared by low pH-treatment of BHA (see *2.2.6 Preparation of low-pH-induced BHA Rosettes, p.84*) and injected over the fetuin-coated sensor surface at different concentrations for 7 min. Dissociation of rosettes from fetuin was measured for 10 min (see Figure 25). However, inspection of the curves shows that the association and

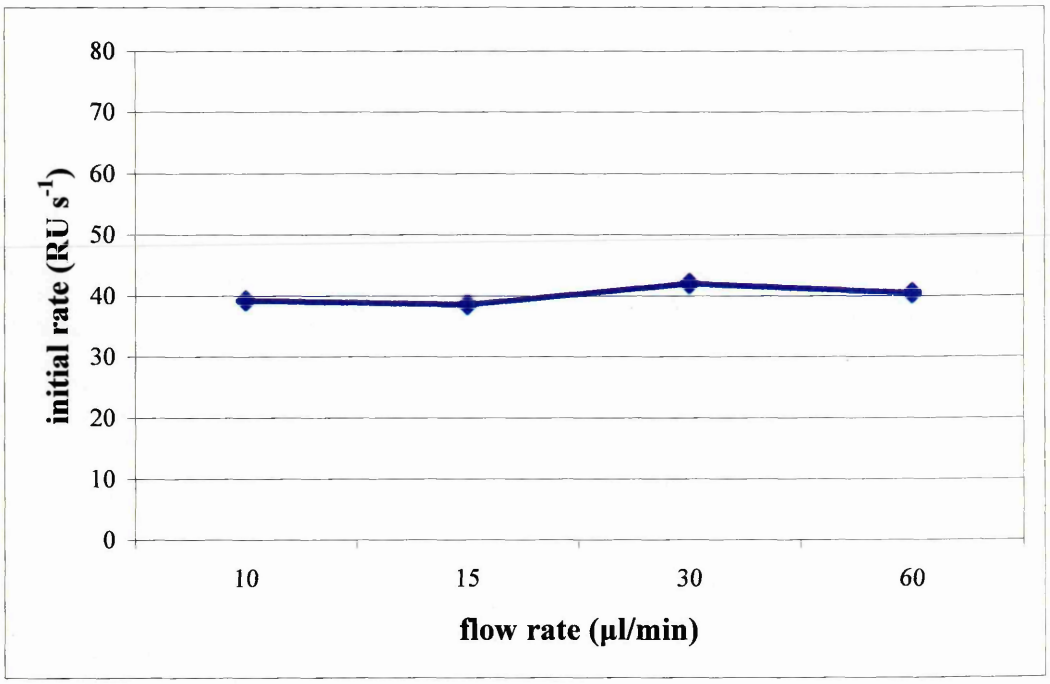


Figure 24 Mass transport test
An increased initial rate (RUs per second) for the complex formation as a result of faster flow rates indicates that the kinetics of the interaction is limited by mass transport. The initial rate was determined for the association curve of X31 virus injected over fetuin at different flow rates. Virus at 0.26 nM was injected over the sensor surface for 1 min. No substantial increase in the initial rate was observed.

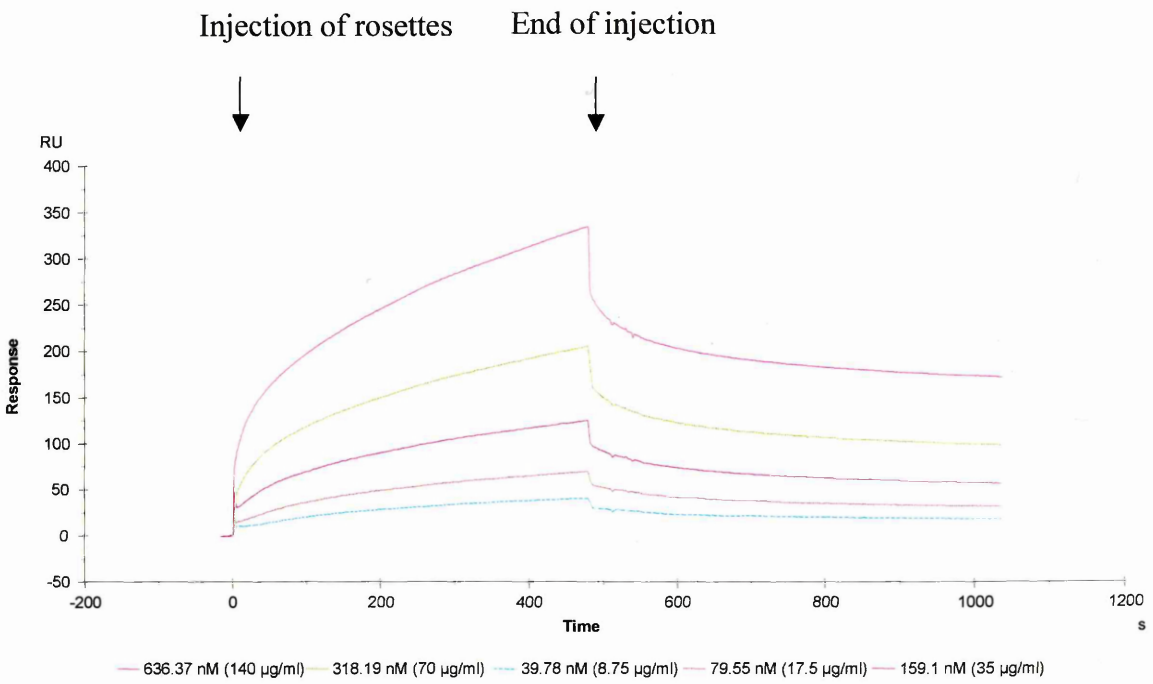


Figure 25 Association and dissociation curves for the interaction of BHA-rosettes with fetuin Rosettes were injected over the sensor surface at five different concentrations for 7 min generating association curves. Upon replacement of sample with buffer, dissociation was observed for 10 min. The association and dissociation phases both contain at least three major kinetic processes.

dissociation phases consist of at least three exponential components, and each of these components makes a substantial contribution to the total reaction amplitude. This makes kinetic analysis to obtain binding constants impossible, since it is not known which association rate constant to associate with which dissociation rate constant. Inspection of the curves obtained for a range of rosette concentrations suggests that the affinity is approximately micromolar, so five or six orders of magnitude weaker than for virus. In regard to these limitations, all subsequent receptor-binding studies were performed with virus as substrate.

3.2.11 Linkage Specificity Studies of Influenza Virus by SPR

Due to the presence of both α(2,3)- and α(2,6)-linked Neu5Ac in fetuin, the affinity data described above does not discriminate between these two components. In order to study the linkage preference (specificity) of influenza virus, rate constants and affinities were determined for derivatised fetuin containing Neu5Ac either in the α(2,3)- or the α(2,6)-linkage (referred to as α(2,3)-fetuin and α(2,6)-fetuin).

3.2.11.1 Preparation of Derivatised Fetuin

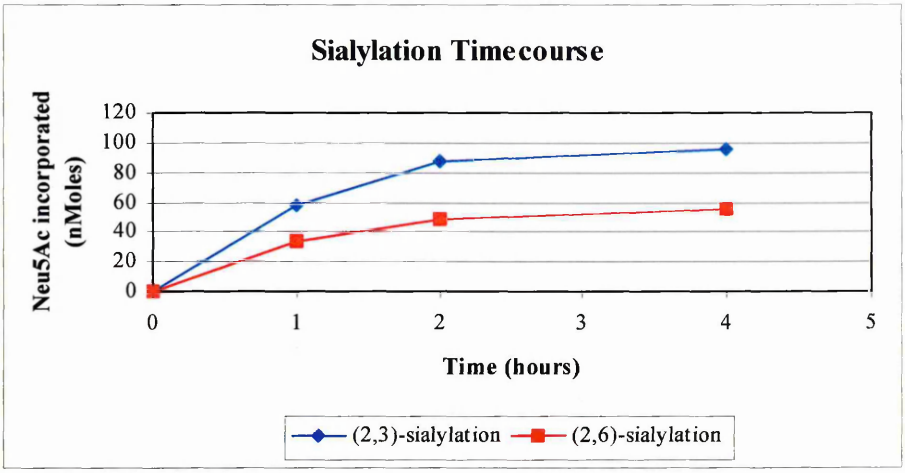
α(2,3)- and α(2,6)-fetuin were prepared by incubation of asialofetuin with CMP-Neu5Ac and linkage-specific sialyltransferases as described in *2.2.16 Preparation of α(2,3)- and α(2,6)-Fetuin as Substrates for Binding Experiments, p.89*. These enzymes covalently bind sialic acid either to Galβ(1,4)GlcNAc in the α(2,6)-linkage or to Galβ(1,4/3)GlcNAc in the α(2,3)-linkage and are specific for N-linked oligosaccharides (Williams et al., 1995). In order to incorporate the same amount of Neu5Ac in either linkage, conditions for saturation of asialofetuin at a specific enzyme concentration were established. Quantitation of transferred Neu5Ac was performed by including a trace amount of ^{14}C-labelled CMP-

Neu5Ac in the reaction mixture. The results of these experiments are shown in Figure 26. Sialylation of fetuin appears to be complete after incubation for 4 hours, as determined in a timecourse experiment (Figure 26a). Then the experiment was performed using varying Neu5Ac concentrations. As can be seen, incubation with 765 nmoles of Neu5Ac leads to saturation of asialofetuin for sialylation in both linkages (Figure 26b). However, incorporation of α(2,6)-linked Neu5Ac was determined to be lower than for α(2,3)-linked Neu5Ac. Therefore, the amount of Neu5Ac was reduced to 153 nmoles for sialylation in the α(2,3)-linkage for preparation of derivatised fetuins for use in SPR assays. Under these conditions, a very similar amount of incorporated Neu5Ac was observed for incubation with both α(2,3)-and α(2,6)-specific transferases (73 and 71 nmoles, respectively).

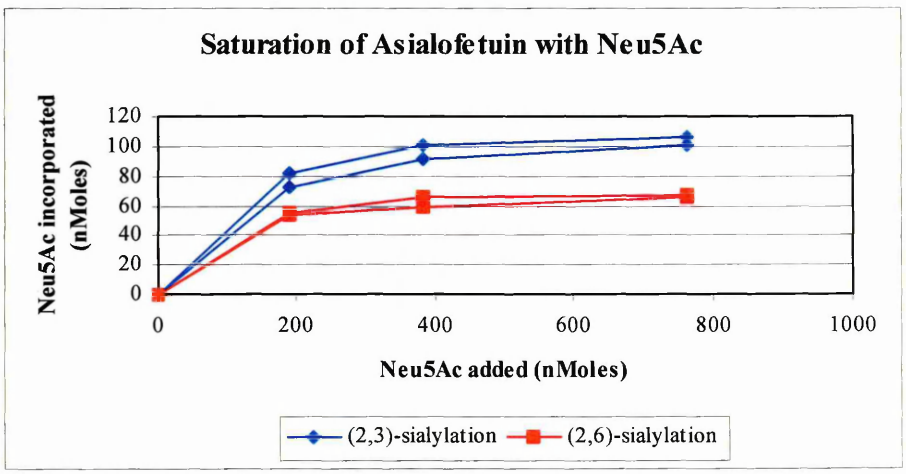
3.2.11.2 Affinity of Influenza Virus for α(2,3)- and α(2,6)- Fetuin

Derivatised fetuin containing Neu5Ac in the α(2,3)- or α(2,6)-linkage was immobilised on the sensor surface as described in *2.2.17 Surface Plasmon Resonance Binding Experiments, p.90*. This led to saturation of the sensor surface, producing a stable signal of ~ 2,000 RUs, which corresponds to a density of fetuin on the sensor surface of ~ 2 ng/mm^2 (Stenberg et al., 1991). Then the interaction of X31 and Gly225Asp virus with the derivatised fetuins was monitored as described above (see Figure 27 for the sensorgrams). As a control, the single-site mutant Leu226Gln was included in the analysis. As described in *1.7.4.2 Correlation between Receptor-Linkage Specificity and Host of Origin, p.39*, residue 226 has been shown to be the major determinant in linkage specificity for the H3 subtype. Figure 28 shows the calculated association rate constant data and Figure 29 the dissociation curves and rate constants. The kinetic data and the affinities of these viruses for α(2,3)- and α(2,6)-fetuin are summarised in Table 2. The specificity index (SI = K_D (α2,3)/K_D (α2,6)) was introduced in order to express the linkage preference (specificity) of

a)



b)



c)

Neu5Ac added (nMoles)	Neu5Ac mean incorporation (nMoles) α (2,3)-linkage	Neu5Ac mean incorporation (nMoles) α (2,6)-linkage
191.25	78	55
382.5	97	63
765	104	67

Figure 26 Preparation of fetuin with Neu5Ac either in the α(2,3)- or the α(2,6)-linkage Neu5Ac was transferred to terminal Gal of asialofetuin by use of linkage-specific sialyltransferases as described in *2.2.16 Preparation of α(2,3)- and α(2,6)-Fetuin as Substrates for Binding Experiments, p.89.* Incorporation of Neu5Ac was quantitated by adding trace amounts of ^{14}C-labelled Neu5Ac and measuring radioactivity of resialylated fetuins. Curves in blue show sialylation with α(2,3)-sialyltransferase and curves in red sialylation with α(2,6)-sialyltransferase **a)** Timecourse for incubation of asialofetuin with 400 nMoles of Neu5Ac to determine the endpoint of the sialylation reaction. **b)** Saturation of asialofetuin with Neu5Ac in the different linkages. Asialofetuin was incubated with different amounts of Neu5Ac for 4 hours and the experiments were performed in duplicates. **c)** Results of sialylation experiment of Figure b), showing that more Neu5Ac is incorporated in the α(2,3)- than the α(2,6)-linkage.

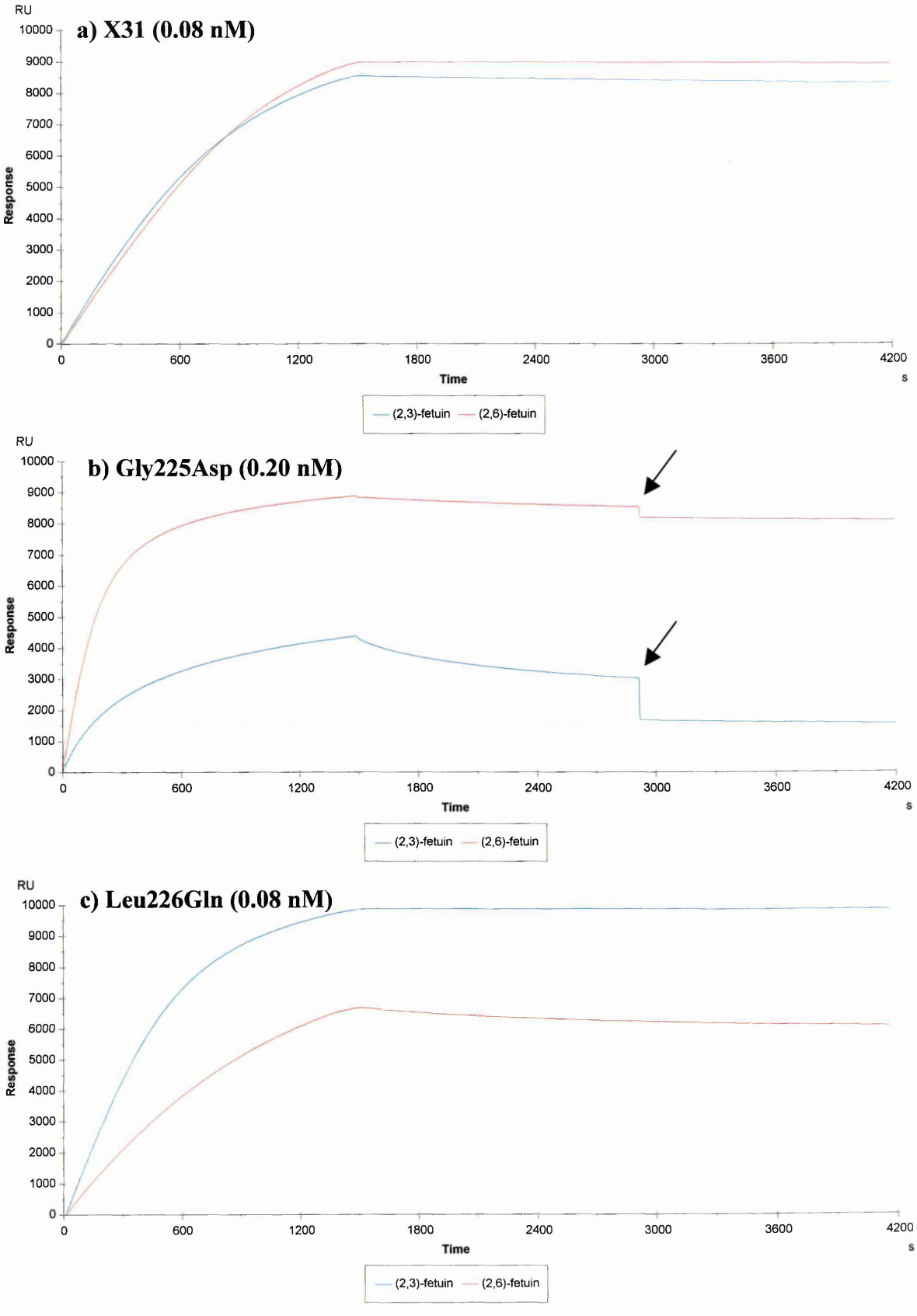
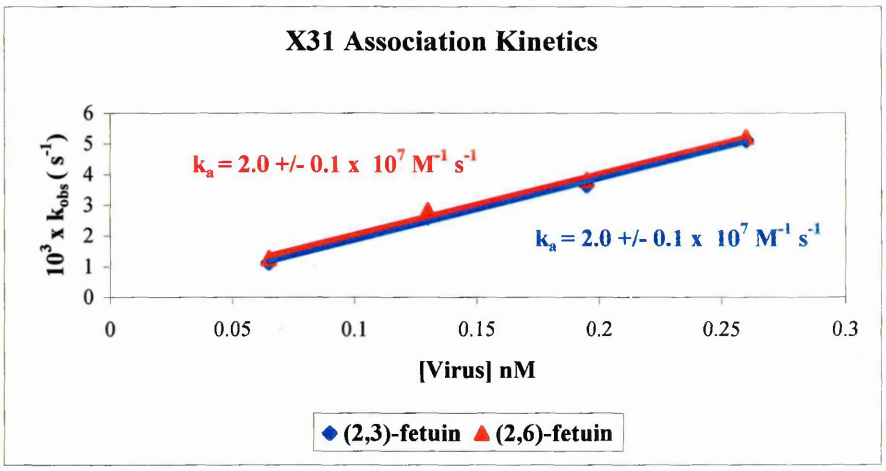
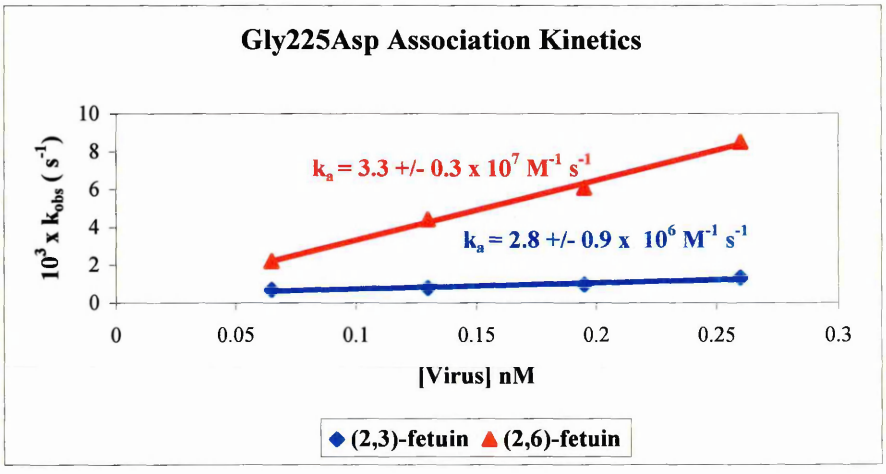


Figure 27 Overlay of association and dissociation curves for the interaction of X31 (a), Gly225Asp (b) and Leu226Gln (c) with resialylated fetuins Viruses were injected over the sensor surface containing α(2,6)-fetuin (red) and α(2,3)-fetuin (blue) for 25 min and dissociation was observed for 45 min. The arrow in **b)** shows a drop of the signal due to buffer refill of the BIAcore pump.

a)



b)



c)

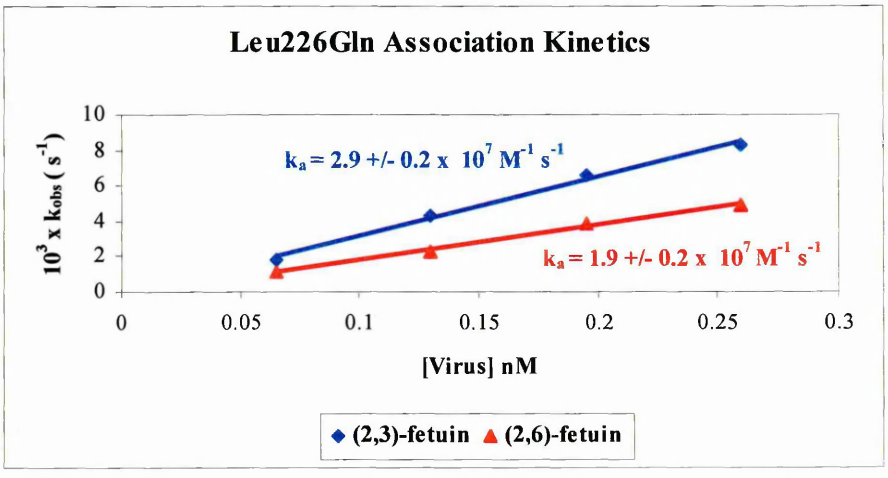
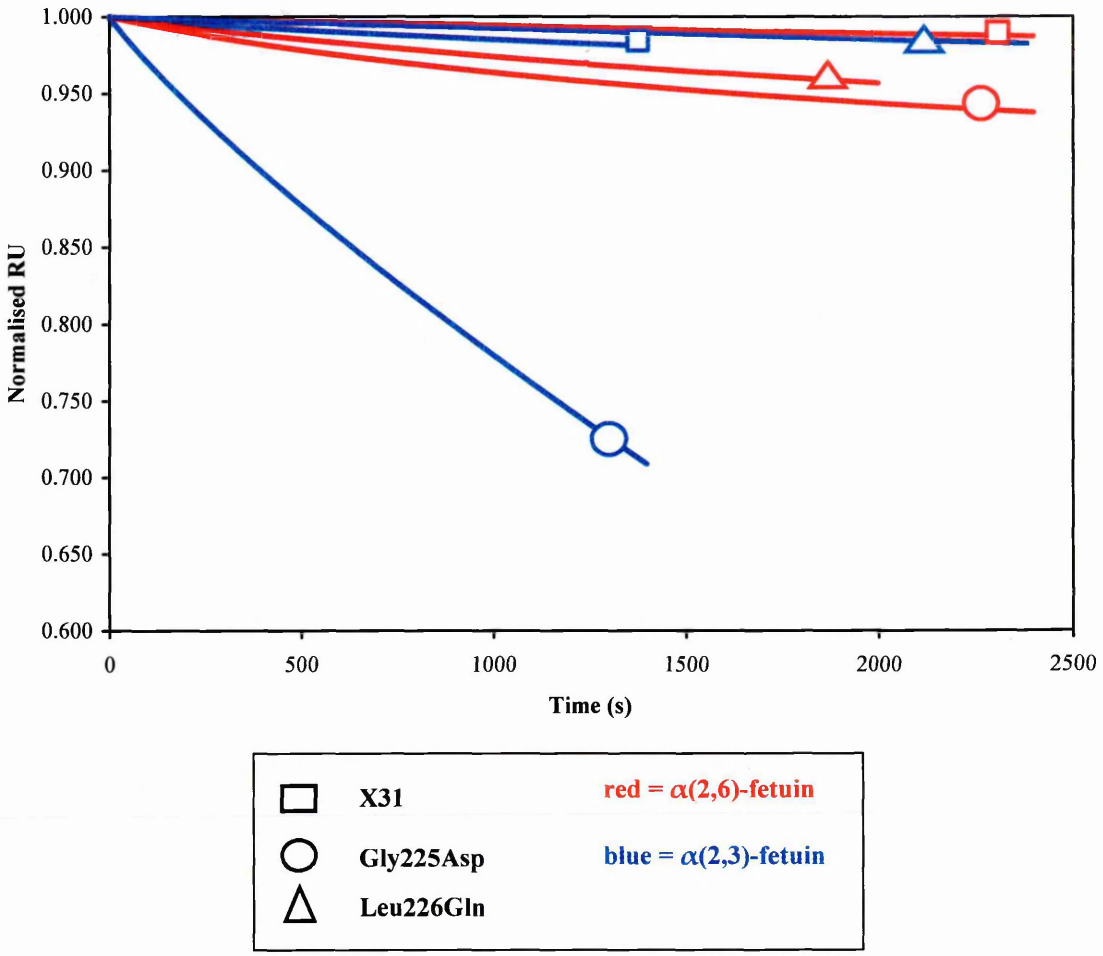


Figure 28 Calculation of association rate constants (k_a) for the interaction of X31 (a), Gly225Asp (b) and Leu226Gln (c) with α(2,6)-fetuin and α(2,3)-fetuin The viruses were injected over the sensor surface containing α(2,6)-fetuin (red) and α(2,3)-fetuin (blue) for 25 min at four different concentrations. The observed rate constant (k_{obs}) was determined for each of the association curves as described in *3.2.5 Calculation of Association Kinetics, p.104.* A plot of k_{obs} against virus concentration generates a straight line with slope k_a.

a) **Dissociation Curves for X31, Gly225Asp and Leu226Gln viruses from (2,3)- and (2,6)-fetuin**



b)

	k_d (2,3)-fetuin (s^{-1})	k_d (2,6)-fetuin (s^{-1})
X31	$9.0 \pm 3.0 \times 10^{-6}$	$1.1 \pm 0.4 \times 10^{-6}$
Gly225Asp	$2.5 \pm 0.7 \times 10^{-4}$	$2.0 \pm 0.6 \times 10^{-5}$
Leu226Gln	$2.1 \pm 0.6 \times 10^{-6}$	$1.6 \pm 0.4 \times 10^{-5}$

Figure 29 Calculation of dissociation rate constants (k_d) for the interaction of X31, Gly225Asp and Leu226Gln with α(2,6)- and α(2,3)-fetuin a) After virus injection for 25 min, dissociation of virus from α(2,6)- and α(2,3)-fetuin was observed for 45 min for one virus concentration (see Figure 27). The dissociation curves were normalised by setting the total reaction amplitude to 1 (see Figure 21 legend) **b)** The k_d was calculated from a single-exponential decay equation as described in *3.2.6 Calculation of Dissociation Kinetics, p.106.*

	α(2,6)-fetuin			α(2,3)-fetuin			
Virus	10^{-6} x k_a ($M^{-1}s^{-1}$)	10^{6} x k_d (s^{-1})	10^{14} x K_D (M)	10^{-6} x k_a ($M^{-1}s^{-1}$)	10^{6} x k_d (s^{-1})	10^{14} x K_D (M)	**Specificity Index (SI)**
X31	20 ± 1	1.1 ± 0.4	5.6 ± 2	20 ± 1	9 ± 3	45 ± 15	8
Gly225Asp	33 ± 3	20 ± 6	61 ± 19	2.8 ± 0.9	250 ± 70	8930 ± 3810	146
Leu226Gln	19 ± 2	16 ± 4	84 ± 23	29 ± 2	2.1 ± 0.6	7.2 ± 2.1	0.09

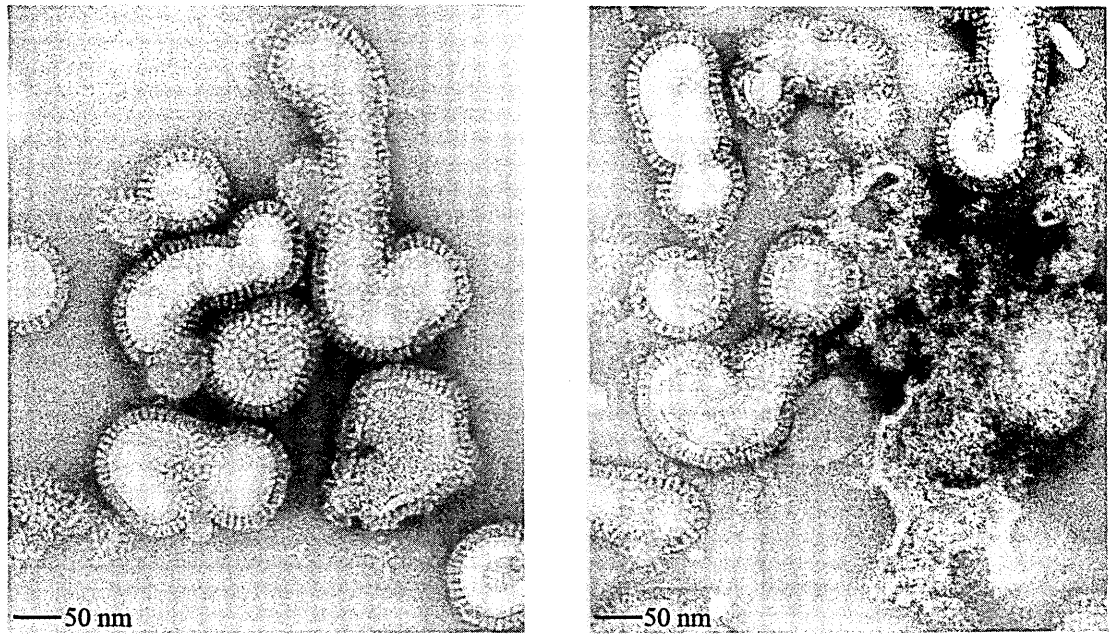
Table 2 Summary of kinetic rate constants and affinities for the interaction of X31, Gly225Asp and Leu226Gln viruses with α(2,6)- and α(2,3)-fetuin The association rate constants (k_a) and the dissociation rate constants (k_d) are shown in Figure 28 and Figure 29, respectively. The affinity of the viruses for the receptor analogues is expressed as $K_D = k_d / k_a$, where by definition high K_D values indicate low affinity. The specificity index (SI) is K_D (α2,3)/K_D (α2,6) and is a measure for the linkage preference of a virus, with < 1 = preference for α(2,3)-fetuin and >1 = preference for α(2,6)-fetuin.

the virus in quantitative terms. As can be seen in Table 2, the tested viruses differed greatly in their specificity. Whereas X31 virus displays an 8-fold higher affinity for Neu5Ac in the α(2,6)-linkage compared to α(2,3), the Leu226Gln mutant binds Neu5Ac in this linkage about 10-fold more weakly than α(2,3)-linked Neu5Ac. The change in specificity results from a ~ 15-fold decrease in affinity for α(2,6)-fetuin and a ~ 6-fold increase in affinity for α(2,3)-fetuin compared to X31. This shift in specificity is in agreement with the reported preference of these viruses to agglutinate derivatised RBC (Rogers et al., 1983a). On the other hand, the mutation Gly225Asp appears to lead to a ~ 200-fold reduction of affinity for the α(2,3)-linkage and to a ~ 10-fold decreased binding strength for the α(2,6)-linkage compared to X31, resulting in a highly α(2,6)-specific virus.

3.2.12 Importance of Quality of Virus Sample Preparation for SPR Experiments

Only pure virus samples consisting of intact virus particles were used in SPR studies. Therefore, every virus was inspected by electron microscopy prior to use. The effect of broken virus particles in a virus sample on affinity measurements was studied by collecting association rate constant data for α(2,6)-fetuin with a batch of intact virus and a batch containing ~ 50% broken particles. Figure 30a shows the electron microscopy pictures and Figure 30b the association kinetics. The apparent k_a for the broken particle batch is about 2-fold lower than for the intact particle batch. The reason for this difference is that as a result of the disruption, the measured NP concentration does not directly correlate with the number of virus particles. The k_d would not be affected by disrupted particles, since it is by definition concentration independent. The use of an intact virus batch therefore reduces errors in the calculation of virus concentration and hence in determination of the association rate constant.

a)



b)

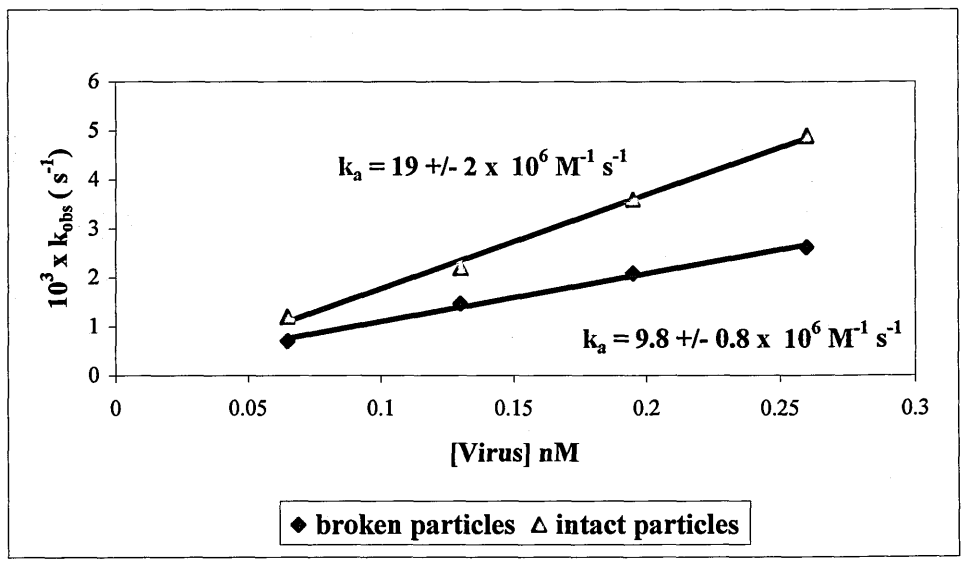


Figure 30 Effect of quality of virus particles on calculation of association rate constants a) Negative-stain electron microscopy pictures of two different batches of Wyoming/3/03. Left = intact virus particles, right = intact and disrupted particles. Disruption occurred during the purification procedure due to instability of the virus particles **b)** Calculation of association rate constants (k_a) for the interaction of intact and broken virus particles with α(2,6)-fetuin. The viruses were injected over the sensor surface containing α(2,6)-fetuin for 25 min at four different concentrations. The observed rate constant (k_{obs}) was determined for each of the association curves as described in *3.2.5 Calculation of Association Kinetics, p.104.* A plot of k_{obs} against virus concentration generates a straight line with slope k_a.

3.3 Discussion

3.3.1 Affinity of Influenza Virus for Fetuin

A novel receptor-binding assay for influenza virus and bovine fetuin using SPR has been established. The interaction with the receptor analogue is specific for Neu5Ac, since no binding to asialofetuin was detected. The generated association and dissociation curves allowed for quantitative analysis of kinetic rates from which the K_D could be calculated. The experiments were shown to be highly reproducible with different batches of the same virus, with only minor differences in the association rate constant obtained from independent experiments. This finding reflects the high resistance of fetuin on the sensor chip with regard to regeneration buffers, efficient activity of the NA inhibitor, insignificant errors in the determination of the virus concentration and fidelity of the BIAcore machine performance. A general disadvantage compared to other receptor-binding assays is the duration of the experiments, since the dataset for every virus is collected separately. Association curves are required to be generated at 4 different virus concentrations in order to calculate an accurate k_a and a separate experiment has to be performed to determine the k_d.

The affinity for the receptor analogue was determined to be very high for a receptor-ligand interaction, with a K_D of ~ 10^{-13} M (see Table 1). The observation that the virus concentration required to produce ~ 50% saturation of the chip is in this concentration range is consistent with a K_D of this magnitude. High affinities (K_D = 10^{-8}-10^{-12} M) have been shown to be a typical feature for the binding of complete virions to cell surfaces (Herrmann et al., 1995). This tight binding is in contrast to the weak interaction of BHA trimers or virus with monovalent receptor analogues, with a K_D of ~ 10^{-3} M as determined by NMR. This difference derives from the fact that the interaction of virus with fetuin in the SPR experiments is multivalent. In the simplest case, the equilibrium

dissociation constant for a multivalent interaction is equal to the K_D for the monovalent interaction raised to the power of n, where n is the valency. The data therefore indicate the simultaneous binding of virus to ~ 4 Neu5Ac moieties in the SPR assay.

The affinity of X31 virus for fetuin as determined by SPR ($K_D \sim 10^{-13}$ M) is significantly higher than the affinity measured in the routinely used solid phase assay ($K_D \sim 10^{-8}$ M) (Gambaryan and Matrosovich, 1992). This discrepancy might reflect the differences in the way these assays are performed. In the solid phase assay the binding strength is determined by measuring the bound fetuin concentration at equilibrium conditions, whereas in the SPR assay, it is calculated from the kinetic constants. However, calculation of the K_D should be the same when determined with either method. Unfortunately, it was impossible to derive a K_D from sensorgram amplitudes because steady-state was not reached on the time scale of these experiments. The main difference between the two assays is the concentration factor in the equations for affinity determination, unbound fetuin in the solid phase study versus unbound virus in the SPR. The much higher affinity determined in the SPR assay is more likely due to the fact that fetuin is immobilised on the sensor surface. As a result, one virus can bind to more than one fetuin molecule, therefore increasing the valency of the interaction. The high local concentration of receptors reflects the conditions of binding of virus to the membrane of a host cell. In contrast, monomeric fetuin is added to immobilised virus in the solid phase virus. Furthermore, the solid-phase assay is complicated by the fact that the virus, which is probed by HRP-labelled fetuin, is immobilised on fetuin-coated wells. This could potentially remove virus from the wells during the experiment and therefore lead to an observed reduction in affinity. Additionally, the presence of the HRP-label might interfere with potential binding sites on the receptor analogue.

3.3.2 Kinetic Rates of the Influenza Virus-Fetuin Interaction

In contrast to other reported assays, SPR is the first to gain insight into kinetic rates of the influenza-receptor interaction. The complex formation between virus and fetuin is fast, with a k_a of ~ 10^7 $M^{-1}s^{-1}$. In contrast, the dissociation rate is extremely slow, resulting in the tight binding of virus to fetuin. A k_d of ~ 10^{-6} s^{-1} indicates that it takes 3 x 10^6 seconds (approximately 35 days) for 95% of the bound virus to dissociate. An accurate calculation of the k_d, however, requires substantial dissociation from the sensor surface. Since it is not feasible to monitor dissociation for that long, the majority of the curve needs to be extrapolated. As a consequence, the confidence of the calculated value of k_d is not as high as for k_a. Nevertheless, even in the worst cases (low k_d values), the dissociation rate constant is unlikely to be wrong by more than a factor of 2-3 and this would not affect the major conclusions of this study.

Analysis of the receptor-binding data revealed heterogeneity in the association and dissociation curves, indicating the occurrence of multiple kinetic processes. Deviations from homogeneous interaction kinetics can be caused by heterogeneous populations of analyte or ligand, or by interference with impurities. A commonly encountered problem of SPR studies is the introduction of heterogeneity by the limited rate of mass transport of analyte to the sensor surface. However, the studies performed in order to assess this possibility exclude mass transport limitation as the cause for the observed heterogeneity in the binding curves. The deviation from monophasic interaction kinetics is more likely to be a result of overcrowding effects of virus at the sensor surface. This has been shown to be a common phenomenon for small immobilised ligands and large analytes at high analyte concentration conditions (Biacore, 2003). These steric crowding effects can be resolved by reducing the density of fetuin on the chip or the concentration of virus. This, however, would lead to a decrease in the observed signal, and therefore limit the detection

range. The component accounting for the majority of the reaction curves was used in the analysis. The validity of this approach was confirmed by the finding that this component varied linearly with virus concentration.

3.3.3 Sensitivity of the SPR Experiments

In order to address whether relatively small differences in affinity can be detected by SPR, the affinity of the single-site mutant Gly225Asp for fetuin was determined and compared to X31 virus. In a binding assay of HA-expressing cells the HA of X31 virus has been shown to bind to RBC about 2-fold more strongly compared to the Gly225Asp mutant (Martin et al., 1998). The obtained SPR data reveal a very similar difference in affinity of these viruses for fetuin, with X31 virus displaying a ~ 3-fold higher binding strength compared to Gly225Asp virus. The kinetic rate constants reveal that the single-site mutant associates slightly faster with fetuin than X31 virus, giving the impression of a higher affinity. However, this is compensated for by a ~ 5-fold decrease in the dissociation rate constant, resulting in reduced binding strength compared to X31 virus.

3.3.4 The Rosette-Fetuin Interaction

Receptor-binding studies were also performed with BHA-rosettes as ligand, in order to determine the effect of valency and particle size on kinetic rate constants and affinity. Previously, a similar assay using SPR has been established to study this interaction, which determined kinetic rate constants of $k_a = 2 \times 10^3\ M^{-1}s^{-1}$ and $k_d = 2 \times 10^{-4}\ s^{-1}$, resulting in a K_D of ~ 10^{-7} M (Takemoto et al., 1996). It was therefore of interest to show how the calculated data compare with the reported kinetic rate constants. However, due to the much higher heterogeneity detected in the association and dissociation curves compared to the virus-fetuin interaction, kinetic analysis of the rosette-fetuin interaction was not possible.

Nevertheless, the fact that high rosette concentrations were required to obtain sensorgrams over the same time ranges as those observed at low virus concentrations does indeed suggest that the association rate constant is much lower for rosettes.

Since mass transport limitations could be ruled out as a factor for the heterogeneity observed in the virus-fetuin interaction, such limitations are not expected to occur when rosettes are used as analyte instead of virus. The reason for this is that transport of analyte to the sensor surface is only affected by the amount of ligand immobilised and the flow rate, both of which are the same in the two experiments. Overcrowding effects at high analyte concentrations are also not likely to occur, due to the smaller size of rosettes compared to virus. Instead, the deviation from single-exponential functions might be a result of the presence of distinct populations of rosettes with regard to the number of HA molecules per particle. This is in agreement with the reported estimate of 6-10 trimers per rosette (Skehel et al., 1982). Since separation of these different populations would be difficult, due to their similarity in size, rosettes are considered unsuitable for affinity measurements by SPR.

3.3.5 Specificity Studies by SPR

An important factor in the evolution of influenza virus in different hosts is thought to be the ability of the virus to distinguish between sialic acid in the α(2,3)- and α(2,6)-linkages. It is therefore of interest to compare the affinities of virus for the receptor determinant in both linkages. The SPR assay described above does not distinguish between the two, due to the presence of both linkages on fetuin. In order to determine the affinity of influenza virus for α(2,3)- and α(2,6)-linked Neu5Ac separately, SPR studies were performed with asialofetuin reconstituted with Neu5Ac in the respective linkages by linkage-specific transferases. When Neu5Ac was provided in excess for both sialylation reactions, more

was transferred to oligosaccharides in the α(2,3)- than the α(2,6)-linkage. This might reflect the difference in specificities of the enzymes for the linkage between Gal and GlcNAc. The α(2,6)-transferase recognises only the β(1,4)-linkage between the two, whereas the α(2,3)-transferase recognises both the β(1,4)- and the β(1,3)-linkage (Williams et al., 1995). Since some N-linked fetuin oligosaccharides have been shown to contain the β(1,3)-linkage, the Gal of these would only be sialylated with Neu5Ac in the α(2,3)- and not the α(2,6)-linkage (Takasaki and Kobata, 1986) (see Figure 17b). Therefore, in order to obtain the same degree of sialylation for incubation with both transferases, the amount of Neu5Ac was reduced for the generation of α(2,3)-fetuin. The same degree of incorporation ensures a constant valency for both α(2,3)- and α(2,6)-fetuin, which would be important for direct comparison of affinities for these receptor analogues.

The SPR assay has been shown to detect up to a 1,000-fold difference in affinities of viruses for the reconstituted fetuins. The ~ 8-fold higher affinity of X31 virus for α(2,6)-fetuin over α(2,3)-fetuin is in agreement with the reported linkage preference of this virus determined by various assays. There are, however, differences in the degree of detected binding of X31 virus to α(2,3)-linked Neu5Ac. Whereas haemagglutination assays using de- and resialylated RBC did not detect binding of X31 virus to α(2,3)-linked Neu5Ac (e.g. Rogers and Paulson, 1983; Daniels et al., 1987), the ability of this virus to recognise this linkage has been shown by haemagglutination assays with paragloboside-reconstituted and derivatised glycoprotein-reconstituted RBC (Suzuki et al., 1986; Suzuki et al., 1987), virus-induced haemolysis of paragloboside-coated RBC (Nobusawa et al., 1991), TLC with paraglobosides (Ryan-Poirier et al., 1998; Masuda et al., 1999), solid-phase assays (Gambaryan and Matrosovich, 1992; Matrosovich et al., 2000) and NMR experiments (Sauter et al., 1989; Hanson et al., 1992). In addition, the structure of X31 HA bound to a receptor analogue containing α(2,3)-linked NeuAc has been solved, providing a structural

basis for the recognition of this linkage (Eisen et al., 1997). The solid phase assays also confirm the enhancement of affinity by multivalency. Compared to the mM affinity of virus for monovalent oligosaccharides, confirming the NMR results, a 1,000-fold increased binding strength was observed upon anchoring multiple copies of the same receptor analogues to a polymeric carrier (Gambaryan and Matrosovich, 1992; Matrosovich et al., 1997). Whereas all the studies reported an α(2,6)-linkage preference for X31, the difference in relative affinity between α(2,3)- and α(2,6)-linked sialic acid differed from 1.5- to 5-fold as determined in assays calculating a K_D. This contrasts with the ~ 8-fold difference observed in the SPR study reported here.

In contrast to X31 virus, the Leu226Gln virus has been shown to switch to preferential α(2,3)-linkage recognition. Again, this finding confirms the linkage preference observed in haemagglutination assays using de- and resialylated RBC (Rogers et al., 1983a; Daniels et al., 1987), solid phase assays (Matrosovich et al., 1993; Matrosovich et al., 1997) and NMR experiments (Sauter et al., 1989). There is, however, an essential difference in how the reported preference for the α(2,3)-linkage is achieved. Whereas Daniels et al. (1987) report the α(2,3)-linkage specificity to be a result of an increase of binding capacity for this linkage, the SPR experiments suggest that the 10-fold higher affinity of Leu226Gln for the α(2,3)-linkage is caused by both an increase in affinity for the α(2,3)-linkage (6-fold) and a decrease in affinity for the α(2,6)-linkage (15-fold) compared to X31. The reduced binding strength for α(2,6)-linked Neu5Ac as a result of the Leu226Gln mutation is supported by the nature of the selection process, inhibition of growth by a receptor analogue containing sialic acid mainly the α(2,6)-linkage (Rogers et al., 1983b). As reported above for X31 virus and α(2,3)-linked Neu5Ac, no binding of Leu226Gln virus to the unfavourable α(2,6)-linkage was detected by haemagglutination assays, unless the RBC were resialylated to contain a substantially higher amount of

α(2,6)-linked Neu5Ac (Daniels et al., 1987). Similarly to X31, the quantitative assays differed in the relative affinities for the two linkages, ranging from 2-fold to greater than 3-fold, which contrasts the 10-fold difference reported here. A summary of the linkage preference data for X31 and Leu226Gln virus reported in the literature is given in Appendix 3, p.257.

In contrast to the shift of preferential linkage-recognition from α(2,6) to α(2,3) of the Leu226Gln mutant, the mutation Gly225Asp appears to result in a substantial decrease in affinity for the α(2,3)-linkage (~ 200-fold compared to X31 virus). This leads to a much higher α(2,6)-linkage preference compared to X31 virus (as described by the specificity index), despite the concomitant decrease in affinity for the α(2,6)- linkage. Although a decrease in affinity for both linkages has been observed before for a virus containing the Gly225Asp together with a Gly135Arg mutation (Laeeq et al., 1997), residue 225 has not been directly implicated in linkage recognition for the H3 subtype. However, a correlation of natural variants containing the same substitution and α(2,3)-linkage preference is described in *Chapter 4*.

General difficulties in affinity data comparison between the described assays are the differences of the substrates used with regard to their monosaccharide composition and linkage between these, valency and receptor density. Although the overall linkage preferences are consistent irrespective of the assay used, the factors described above influence both the calculation of absolute and relative affinities. Furthermore, it is unknown how well these experiments resemble the *in vivo* virus-receptor interaction at the cell surface. Difficulties in the understanding of this process are the lack of information on receptor density, distance from the cell surface, accessibility, valency and the identity of utilised receptors.

4 Changes in Receptor-Binding Properties of H3 and H1 Subtype Viruses in Relation to Antigenic Drift

4.1 Introduction

4.1.1 Identification of Antigenic Drift Variants

HA is subject to antigenic drift through human immune surveillance, leading to the emergence of antigenic variants with epidemic potential *(see 1.7.5 Antigenic Variation, p.52)*. In order to update the vaccine composition for novel variants the global influenza surveillance network routinely monitors the antigenic, genetic and epidemiological properties of circulating viruses. Ferrets are naturally susceptible to human influenza viruses and provide a suitable animal model for the study of immunity in humans due to their similarities in regard of disease symptoms (Smith et al., 1933) and elicited immune response. Their post-infection antibodies are subtype-specific (WHO, 1980) and have been shown to detect antigenic variation within the same subtype (e.g. Hirst, 1943b; Pereira et al., 1964; Both et al., 1983b; Raymond et al., 1986). The most widely used method for measuring the interaction of virus with antibody is the HI assay (Hirst, 1942b; WHO, 2005c). This technique is based on the interference of bound antibody with the interaction of the virus with receptors on RBC. The advantage of this competition-based assay in the detection of antigenic variants is that only the viruses resistant against potentially neutralising antibodies are detected. However, the involvement of receptor-binding properties in HI assays also presents difficulties in the analysis of results. The failure of certain viruses to react with antibodies as judged by an HI test might not necessarily reflect differences in antigenicity but be the result of altered receptor-binding properties (e.g. Daniels et al., 1987). A further drawback of the HI test is its reported limitation to the

detection of only major drift variants (Smith, 2003). Efforts are currently being made to improve the sensitivity of reliable measurements by the introduction of mathematical methods for the analysis of results (Smith et al., 2004).

Antigenic differences are also readily detected in virus neutralisation tests (Smith et al., 1933; WHO, 2005b). However, these experiments are too labour-intense for routine use and, as for the HI test, the same problems with receptor-binding properties may be encountered. In contrast, immunodiffusion (Schild, 1970; Schild et al., 1972; Hornbeck, 2001), complement fixation (CF) (Lief and Henle, 1959) and ELISA assays are not affected by receptor-binding properties of the viruses used. Although these methods do not discriminate between neutralising and non-neutralising antibodies, they can assist in the analysis of the results obtained in HI tests.

4.1.2 Molecular Mechanism for Antigenic Drift

The ultimate goal of antigenic and genetic studies of HA is to understand the underlying patterns of HA evolution during replication in humans so as to be able to predict the next antigenic variant with epidemic potential. Therefore, great efforts are being made to detect HA residues under positive selection and to predict the fitness of a potential new variant by mathematical and computational methods (Bush et al., 1999a; Lee and Chen, 2004; Smith et al., 2004). However, the molecular mechanism of the appearance of antigenic drift strains is not completely understood. Therefore, the timing and speed, as well as the nature of antigenic variation have remained unpredictable. Several mechanisms for the escape from neutralisation by human polyclonal antibodies have been proposed. Studies with mice have shown that neutralising antibodies generated as a result of intranasal infection are directed only against a limited number of antigenic sites (Smith et al., 1991; Patera et al., 1995). Serological studies have also indicated a similar restricted antibody-repertoire in

humans (Natali et al., 1981), rabbits and ferrets (Laver et al., 1981; Cleveland et al., 1997). Therefore, inhibition of binding by the predominant antibodies by substitutions at immunodominant epitopes might be sufficient for evasion of immune recognition. However, it has been shown that the specificity of produced antibodies varies between individuals (Wang et al., 1986; Nakajima et al., 2000). This suggests that sequential epitope changes in HA are necessary for a virus to be able to re-infect members of a population so as to evade herd immunity. The observation that drift variants of epidemiological importance usually contain ≥ 4 amino acid changes located in ≥ 2 of the antigenic sites (Wilson and Cox, 1990) supports this mechanism. Furthermore, changes in residues other than those targeted by antibodies might play a role in immune-evasion. Namely, since antibodies compete with cells for binding to virus, changes in the interaction with cellular receptors have been proposed to present an alternative mechanism for antibody-escape. Evidence for this is provided by the selection of receptor-binding variants (adsorptive mutants) by monoclonal and polyclonal antibodies (see *1.7.6 Interrelationship between Antigenic Variation and Receptor-Binding Properties, p.58*). Furthermore, viruses with substitutions in the RBS are frequently isolated, many of which become fixed in the evolutionary line (e.g. Nobusawa et al., 2000; Medeiros et al., 2001). In addition, genetic studies have shown that a number of residues identified to be under positive selection in H3 HA are in the RBS (residues 135, 138, 190, 194 and 226) (Bush et al., 1999b), and are therefore likely to affect receptor-recognition.

The reason for the selection of adsorptive mutants is not clear. It has been suggested that antigenic changes might be limited by the need to maintain the structure of HA (Both et al., 1983b). Direct evidence for structural constraints is supported by a study showing that a limited number of mutations in immunodominant sites provide constraints for further antigenic change and the respective antibodies select for receptor-binding

variants instead (Temoltzin-Palacios and Thomas, 1994). Alternatively, it has been suggested that sub-neutralising levels of antibodies (Fazekas de St. Groth, 1977; Yewdell et al., 1986; Temoltzin-Palacios and Thomas, 1994) or antibodies of reduced affinity (Laeeq et al., 1997) play a role in this process.

In summary, the implication of changes in receptor-binding specificity/affinity in immune surveillance highlights the importance of monitoring these properties, in addition to antigenicity, for circulating viruses. The following study compares the receptor-binding properties of a panel of viruses of the H1 and H3 subtypes in relation to antigenic drift. These should lead to a further understanding of the interrelationship between these two properties and complement the available surveillance data.

4.1.3 Host Cell-Mediated Variation

Characterisation of antigenic and receptor-binding properties of circulating viruses involves the propagation of the original clinical specimen in laboratory host cell systems. However, initial adaptation to certain host cells, most importantly to growth in the allantoic cavity of eggs (egg-adaptation), has been shown to select for variants with distinct receptor-binding and/or antigenic properties. This phenomenon is known as host cell-mediated variation and was first noted by Burnet and Bull (Burnet and Bull, 1943), who showed that viruses repeatedly cultivated in eggs differed from the original isolate in their agglutination capacities of chicken RBC. Further evidence for egg-mediated variation was provided by studies with influenza B virus showing that viruses with altered antigenic properties can be selected upon propagation in eggs compared with the same isolate grown in MDCK cells (Schild et al., 1983). Sequence analysis revealed that the antigenic change was associated with the loss of a specific glycosylation site at the distal tip of HA (Robertson et al., 1985). In contrast to eggs, mammalian tissue culture cell lines, such as

the routinely used MDCK and Vero cells, appear to select for variant viruses far less frequently (Katz and Webster, 1992; Govorkova et al., 1996). Sequencing of individual PCR-amplified clones has shown that virus derived from MDCK cells is representative of virus present in the clinical isolate, whereas egg-adapted virus can contain several different variants (Robertson et al., 1990; Robertson et al., 1991). Exceptions to this are baby hamster kidney (BHK) cells, which, like eggs, often select for receptor-binding variants (Govorkova et al., 1999).

Since propagation of MDCK-isolates in eggs often leads to the selection of variants but not the other way around, it was suggested that eggs provide the more restrictive system for virus replication. Upon initial egg-adaptation, influenza virus usually grows to high titres in the allantoic cavity. It has long been recognised that initial passage in the amnion prior to propagation in the allantoic cavity leads to higher efficiency of virus isolation (Hoyle, 1968b). However, despite the observed unrestricted growth in this host cell system, repeated cultivation in the amnion also leads to the selection of variants (Robertson et al., 1993; Ito et al., 1997b). The reason for this has been suggested to be exposure of the amniotic virus to the allantois through a hole between the two compartments caused by inoculation needles.

Pairwise sequence comparison of MDCK- and egg-grown viruses has identified the changes at a number of residues implicated in egg-adaptation. Table 3 shows amino acid substitutions most frequently associated with this process for H1 and H3 subtype viruses (see legend for references). A number of identified changes are located in antigenically important areas, explaining why host cell-mediated variants often display altered antigenic properties (Schild et al., 1983; Oxford et al., 1987). However, the majority of the implicated substitutions were found to cluster around the RBS, which suggested their selection on the basis of altered receptor-recognition. Indeed, it was noted that egg-adapted

Table 3 Residues reported to be directly associated with egg-adaptation for H1 and H3 viruses
Changes at residues in red abolish a glycosylation site. Residues in blue have been suggested to be under positive selection in the human population (Bush et al., 2000). Residues in brackets occurred in conjunction with some of those listed above and may also be associated with egg-adaptation.

References for substitutions in H3 viruses: (Katz et al., 1987; Katz and Webster, 1988; Wang et al., 1989; Katz et al., 1990; Katz and Webster, 1992; Meyer et al., 1993; Robertson, 1993; Rocha et al., 1993; Hardy et al., 1995; Gubareva et al., 1994; Ito et al., 1997b)
References for substitutions in H1 viruses: (Robertson et al., 1987; Robertson et al., 1991; Robertson, 1993; Rocha et al., 1993)

Affinity change as a result of egg-adaptation is compared to virus propagated in MDCK cells without amino acid substitution in HA (Gambaryan et al., 1999; Mochalova et al., 2003). All substitutions, for which affinity changes are reported, have an increased binding strength for chorio-allantoic membrane cells. ↑ = increase in affinity, ↓ = decrease in affinity, 3'SL = Neu5Acα(2,3)Galβ1,4Glc, 6'SL = Neu5Acα(2,6)Galβ1,4Glc, 6'SLN = Neu5Acα(2,6)Galβ1,4GlcNAc, PAA = polyacrylamide-carrier (for use of polyvalent receptor analogues). Dextran sulphate was used as a substrate to measure affinity for negative charge.

H3 viruses			H1 viruses		
Residue Change		**Affinity Change**	**Residue Change**		**Affinity change**
137	Tyr → Ser		129	Asn → Lys	
138	Ala → Thr		156	Glu → Lys	
145	Asn → Lys	↓ 6'SL (2.5-fold) and ↑ dextran sulphate (>10-fold)	163	Asn → Lys/Ser	↓ 6'SLN (4-fold), thought to resolve steric hindrance with distal parts of α(2,3)-linked receptor-analogues
155	Tyr → His				
156	Glu → Lys	↓ 6'SLN (12-fold) and ↑ dextran sulphate (>20-fold)	187	Asn → Lys	↑ dextran sulphate (50-fold)
158	Glu → Lys		189	Glu → Lys	↑ dextran sulphate (250-fold)
159	Ser → Arg		190	Asp → Asn	↑ 3'SL (2-3-fold) and ↓ 6'SLN (2-2.5-fold) and ↑ dextran sulphate (50-fold)
144	Val → Ala	} changes occurred together			
159	Tyr → His		225	Asp → Gly	↑ 3'SL (2-6-fold) and ↓ 6'SLN (0-2.5-fold) ↑ 3'SL-PAA (> 500-fold)
185	Ser → Ile				
186	Ser → Ile	↑ 3'SL (3-5-fold)	225	Asp → Asn	no change for 3'SL and 6'SLN
193	Asn → Lys	} changes occurred together	226	Gln → Arg	↑ 3'SL-PAA (25-fold) and ↓ 6'SLN-PAA (> 300-fold)
229	Arg → Gly		227	Glu → Arg	
194	Leu → Ile				
219	Ser → Tyr		(138	Ser → Ala)	
226	Leu → Gln				
246	Asn → Ser				
or					
248	Thr → Ile				
(126	Asn → Asp)				
(163	Ala → Val)				

viruses displayed an increased affinity for cells of chorio-allantoic membranes (CAM), the major site of replication in eggs, compared to MDCK cell-grown virus (Gambaryan et al., 1999). Staining of cells with linkage-specific lectins indicated that, whereas chorio-allantoic and BHK cells contain predominantly α(2,3)-linked sialic acid (Ito et al., 1997b; Govorkova et al., 1999), human tracheal cells express mainly α(2,6)-linked sialic acid (Baum and Paulson, 1990; Couceiro et al., 1993). Since increased binding of many egg- and BHK-adapted viruses to receptor analogues containing α(2,3)-linked Neu5Ac was observed compared to MDCK cell-grown virus, it was suggested that variants are selected because the affinity of the non-adapted human viruses for the α(2,3)-linkage is too low (Gambaryan et al., 1997; Ito et al., 1997b; Govorkova et al., 1999). In contrast, MDCK and amniotic cells contain similar amounts of α(2,3)- and α(2,6)-linked sialic acid (Govorkova et al., 1996; Ito et al., 1997b), explaining unrestricted growth of human viruses in these cells. However, VERO cells contain predominantly α(2,3)-linked sialic acid but, in contrast to eggs and BHK cells, appear not to select for variants (Govorkova et al., 1996). This indicates that either the number of α(2,6)-linked sialic acids on VERO cells is sufficient for infection or that additional host-cell factors, yet to be identified, might play a role in host-cell mediated variation.

Table 3 shows the single-site substitutions of egg-adapted variants for which the effect on receptor-binding properties has been directly tested by comparison with MDCK-grown counterparts (Gambaryan et al., 1999; Mochalova et al., 2003). Confirming earlier observations, many of the substitutions lead to an increased affinity for α(2,3)-linked sialic acid. Other substitutions, which introduce a positively charged amino acid in HA_1, have been suggested to enhance affinity for negatively charged receptors on host cells by non-specific ionic interactions, based on the interaction with dextran sulphate (Gambaryan et al., 1999). Furthermore, carbohydrates at the membrane-distal part of HA have been

shown to generally interfere with the interaction of HA with cellular receptors by steric hindrance (Deom et al., 1986; Gunther et al., 1993; Matrosovich et al., 1997; Ohuchi et al., 1997; Abe et al., 2004). Therefore, substitutions leading to the loss of carbohydrate attachment sites also have the ability to increase receptor-affinity. It has been suggested, for example, that the loss of a carbohydrate at 163 as a result of egg-adaptation for H1 viruses would lead to a decrease of steric hindrance with more distant parts of receptors containing α(2,3)-linked sialic acid (Gambaryan et al., 1999).

Host cell-mediated selection of variants needs to be taken into consideration when receptor-binding and antigenic properties of clinical isolates are studied. Importantly, since vaccine candidates are still propagated in eggs, it needs to be ensured that their antigenic properties reflect those of the viruses circulating in the human population. Therefore, the HA should be sequenced directly from a clinical sample prior to cultivation of the virus in the laboratory, so that the predominant virus in the sample can be determined (Robertson et al., 1990; Robertson et al., 1991). Furthermore, the parallel propagation in eggs and MDCK/VERO cells and the monitoring of sequence changes in HA can assist in the detection of host cell-mediated variation. However, it is often difficult to trace back egg-adaptation mutations, since many viruses are subjected to cultivation in eggs prior to sequencing and because information of the passage history of the viruses used is often incomplete.

All the viruses used in the following study were egg-adapted and were further propagated in eggs due to the general high yield and ease of purification from this host cell system. Furthermore, viruses isolated from eggs generally display a more homogeneous spherical morphology (Burnet and Lind, 1957; Choppin et al., 1960) than those propagated in mammalian tissue culture cells (L. Calder, NIMR, London, personal communication). Since a virus population of different sized particles is likely to result in heterogeneous

association and dissociation kinetics (see *3.3.4 The Rosette-Fetuin Interaction, p.126*), a homogeneous morphology of the virus sample was considered to be important for SPR studies. The sequence data of the original clinical isolates was either not readily available, or, in the case of viruses isolated before PCR techniques became available, non-existent. The exact passage history of these viruses in eggs is also unknown. Whereas the passage number in eggs was estimated to be in the range of 4-8 for viruses isolated since 1980 (WIC, personal communication), information for the earlier viruses is missing. Therefore, although it was not possible to clearly identify egg-adaptation mutations, their presence was addressed by comparison of residues that had previously been associated with this process (see Table 3). The difficulty with this approach is, that in the course of HA evolution, substitutions at different residues can become selected by egg-adaptation. For example, it has been shown that cultivation of H3 isolates of 1994 in eggs often led to variants at residues 194 and 226, rather than at residues 156 and 186 of H3 isolates of 1988 (Hardy et al., 1995), which complicates egg-adaptation analysis. Furthermore, a number of residues that have been implicated in egg-adaptation for the H3 subtype have also been suggested to be under positive selection in the human host, which indicates the possibility of selection by escape from immunity as well (Bush et al., 2000). Identification of genuine egg-adaptation mutations for the isolates used in this study was therefore not possible.

4.2 Receptor-Binding Properties of H3 Subtype Viruses

4.2.1 Antigenic and Phylogenetic Relationships

Antigenically distinct H3 viruses from 1994-2003 were tested for their receptor-binding properties using SPR. The H3 subtype viruses emerged in humans in 1968, causing the Hong Kong pandemic, and have continued to circulate until today. The viruses included in the study were a selection of vaccine strains of 1994 (Johannesburg/33/94) until 1999

(Panama/2007/99), and the novel antigenic variants that appeared in 2002 (Fujian-like viruses). Following their emergence, these latter viruses have gradually increased in prevalence compared to Panama/2007/99-like viruses and since 2004 have replaced this virus as the H3 vaccine component. Since the reference virus Fujian/411/02 grew very poorly in eggs, this virus was not included in the study. Table 4 shows the antigenic characteristics based on HI tests using post-infection ferret sera. Sydney/5/97 and Panama/2007/99 are antigenically closely related but different to Johannesburg/33/94. The remaining viruses are clearly distinguished from Panama/2007/99 by reactivity with ferret sera and are antigenically related (Fujian-like viruses). Although differences can be seen in reactivity with ferret sera for the Fujian-like viruses, they could not be clearly separated into different antigenic groups. Figure 31 shows the phylogenetic relationship of the viruses used in this study including a number of additional viruses isolated from 1992-2003. As can be seen, the emergence of the antigenic variants coincided with the appearance of phylogenetically distinguishable variants. Although not apparent from Figure 31 due to the limited number of isolates, H3 viruses have evolved as a single lineage since their introduction into the human population in 1968. Therefore, genetically distinct viruses evolve directly from the preceding circulating viruses (Hay et al., 2001). However, it has been shown that the rate of HA antigenic evolution does not necessarily reflect the rate of genetic evolution. Although the rate at which substitutions occurred between 1997-2002 was similar to that before 1997 and led to the emergence of phylogenetically distinct lineages, the resulting amino acid changes did not result in significant antigenic changes (Hay et al., 2001). This is reflected in only one change in the vaccine component for the H3 viruses from 1997-2001. In contrast, the antigenic drift rate was high from 1968-1997, accounting for 18 changes in the recommended vaccine composition.

	Haemagglutination inhibition titre								
	Post-infection ferret sera								
Virus	Aich/2/68	Jhb/33/94	Syd/5/97	Pa/2007/99	Ku/102/02	Chi/1/03	Wy/3/03	UK/1861/03	CC/28/03
Aichi/2/68 (X31)	**1280**	<	<	<	<	<	<	<	<
Johannesburg/33/94	<	**640**	<	<	<	<	<	<	<
Sydney/5/97	<	<	**5120**	2560	80	320	160	160	160
Panama/2007/99	<	<	5120	**2560**	160	640	320	160	320
Kumamoto/102/02	<	<	<	640	**5120**	320	2560	1280	5120
Chita/1/03	<	<	<	640	2560	**1280**	2560	320	1280
Wyoming/3/03	<	<	<	640	5120	320	**5120**	1280	5120
United Kingdom/1861/03	<	<	<	80	320	80	640	**1280**	640
Christchurch/28/03	<	<	<	160	1280	1280	1280	320	**1280**

Table 4 Antigenic analysis of H3N2 viruses Haemagglutination inhibition (HI) reactions of post-infection ferret sera with influenza H3N2 viruses. HI titres represent the reciprocal of the highest dilution of ferret sera inhibiting 8 haemagglutination units of virus. < represents titres less than 40, homologous titres are in bold.

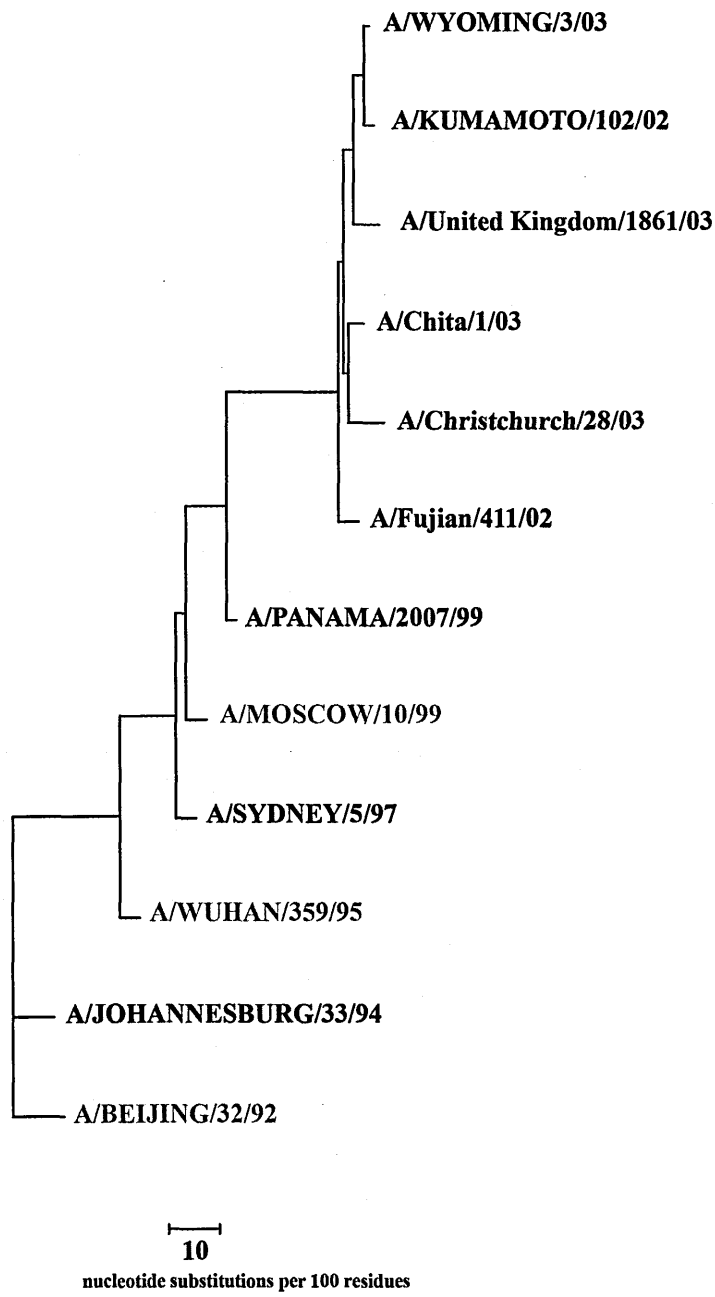


Figure 31 Phylogenetic comparison of H3 HA genes
The sequences of nucleotides 78-1061 encoding the HA_1 domain were compared using *Phylogenetic Analysis Using Parsimony*, version 4.0 (Swofford and Olsen, 1990). Due to the limited number of strains used in this study, three additional viruses were used to construct the tree (indicated in blue). The lengths of the horizontal lines are proportional to the number of nucleotide differences, as indicated by the bar. Vaccine strains are indicated in capital letters.

4.2.2 Affinity and Specificity Studies by SPR

The affinity of the antigenically distinct viruses for α(2,6)- and α(2,3)-linked sialic acid receptor analogues was determined as described in *Chapter 3*, with the kinetic rate constants calculated from 2 independent experiments. The viruses that were used to establish the conditions of the SPR experiments (X31 and its single-site mutants Leu226Gln and Gly225Asp) were included in the receptor-binding studies as controls, with X31 also representing the first human H3 subtype virus isolated at the beginning of the 1968 Hong Kong pandemic (Aichi/2/68). Furthermore, Dk/Ukraine/1/63 was included to compare the receptor-binding properties of an avian virus to those of human viruses. A Dk/Ukraine/1/63-like virus is thought to be the progenitor from which the HA of the pandemic virus Aichi/2/68 was derived after reassortment with a human virus (Laver and Webster, 1973; Fang et al., 1981; Bean et al., 1992).

Table 5 shows the calculated rate constants and affinities of the H3 viruses for α(2,3)- and α(2,6)-fetuin and the specificity index. Since the binding strength of viruses for the receptor analogues varied up to ~ 3,000-fold, the affinity is displayed in log-scale in Figure 32 for ease of comparison. Most of the human viruses used in this study display a higher affinity for α(2,6)-fetuin compared to α(2,3)-fetuin, whereas the avian virus and the single-site mutant Leu226Gln preferentially recognise α(2,3)-fetuin. This finding is in agreement with the reported specificity of human and avian H3 subtype viruses (see *1.7.4.2 Correlation between Receptor-Linkage Specificity and Host of Origin, p.39*). However, two of the viruses, Johannesburg/33/94 and Panama/2007/99, appear not to distinguish significantly between α(2,3)- and α(2,6)-fetuin, and these viruses are therefore classified as "dual"-linkage-specific. Generally, the viruses can be separated into two groups with regard to recognition of the α(2,3)-linkage. Whereas X31 and the viruses isolated from 1994-1999 reveal considerable binding strength for α(2,3)-fetuin compared

	α(2,6)-fetuin			α(2,3)-fetuin			
Viruses	10^{-6} x k_a ($M^{-1}s^{-1}$)	10^{6} x k_d (s^{-1})	10^{14} x K_D (M)	10^{-6} x k_a ($M^{-1}s^{-1}$)	10^{6} x k_d (s^{-1})	10^{14} x K_D (M)	**Specificity Index**
X31 (Aichi/2/68)	20 ± 1	1 ± 0.4	**6 ± 2**	20 ± 1	9 ± 3	**45 ± 15**	7.5
Johannesburg/33/94	21 ± 1	7 ± 3	**33 ± 14**	26 ± 2	10 ± 4	**39 ± 16**	1.2
Sydney/5/97	17 ± 3	1 ± 0.6	**8 ± 3.9**	19 ± 2	7 ± 3	**37 ± 16**	4.6
Panama/2007/99	11 ± 1	1 ± 0.4	**11 ± 4**	16 ± 2	3 ± 0.8	**16 ± 5**	1.5
Kumamoto/102/02	5 ± 1.2	70 ± 25	**1,400 ± 620**	<	>	>	>
Chita/1/03	35 ± 6	25 ± 8	**71 ± 25**	2 ± 0.6	230 ± 70	**15,000 ± 7000**	210
Wyoming/3/03	19 ± 1	60 ± 15	**320 ± 83**	<	>	>	>
United Kingdom/1861/03	16 ± 2	4 ± 1	**25 ± 7**	<	>	>	>
Christchurch/28/03	17 ± 2	1 ± 0.4	**7 ± 2.5**	2 ± 0.6	520 ± 150	**24,000 ± 9,800**	3400
Dk/Ukraine1/63	20 ± 3	100 ± 30	**510 ± 180**	21 ± 3	2 ± 1	**9 ± 4.9**	0.02
Leu226Gln	19 ± 2	16 ± 4	**84 ± 23**	29 ± 2	2.1 ± 0.6	**7.2 ± 2.1**	0.09
Gly225Asp	33 ± 3	20 ± 6	**61 ± 19**	3 ± 0.9	250 ± 70	**9,000 ± 3,800**	150

Table 5 Kinetic rate constants and affinities for the interaction of H3N2 viruses with α(2,6)- and α(2,3)-fetuin The association rate constants (k_a) and dissociation rate constants (k_d) were determined by SPR. Virus at four different concentrations was injected over the BIAcore sensor chip containing immobilised derivatised fetuins. Kinetic rate constants were determined as described in *Chapter 3* and the affinity expressed as k_d/k_a (K_D). High K_D values indicate low affinity. < and > indicate that values were below detection limit. The specificity index (SI) is K_D (α2,3)/K_D (α2,6) and is a measure for the linkage preference of a virus, with < 1 = preference for α(2,3)- and >1 = preference for α(2,6)-fetuin. α(2,6)-specific viruses are indicated in red, α(2,3)-specific viruses in blue and "dual"-specific viruses in black. The control viruses X31, Gly225Asp and Leu226Gln were included in the Table.

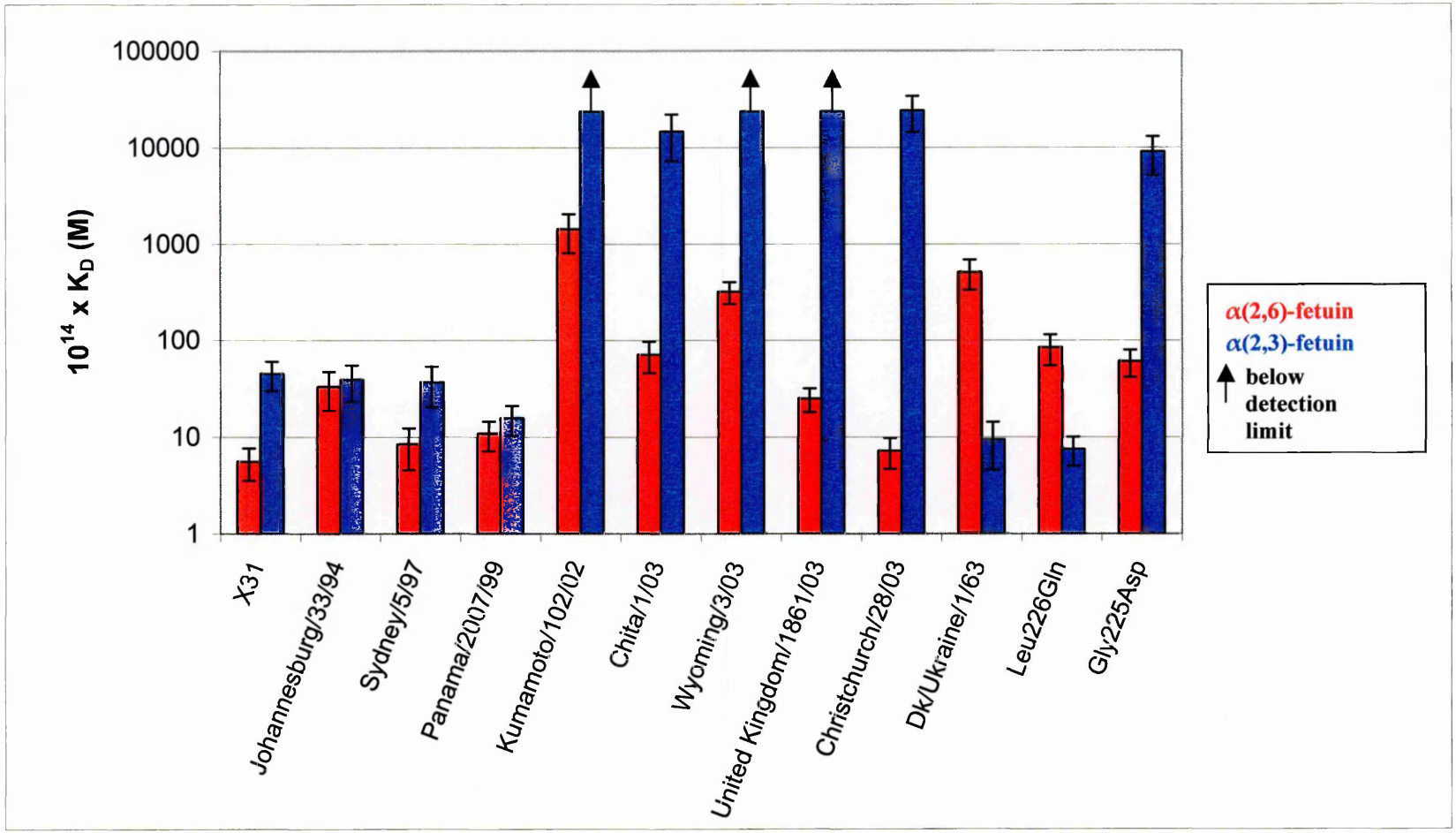


Figure 32 Affinities of H3N2 viruses for α(2,6) and α(2,3)-fetuin The calculated K_D values shown in Table 5 are displayed in log-scale. High values indicate low affinity. Black arrows indicate that the affinities were too low to be detected by SPR. The control viruses X31, Gly225Asp and Leu226Gln were included in the Figure.

to α(2,6)-fetuin (only up to 8-fold affinity difference), all the Fujian-like viruses show a big decrease in affinity for the α(2,3)-linkage. For example, Chita/1/03 and Christchurch/28/03 bind α(2,3)-fetuin ~ 400-fold and ~ 650-fold more weakly than Sydney/5/97, respectively. Notably, the affinity of Wyoming/3/03, Kumamoto/102/02 and United Kingdom/1861/03 for the non-preferred receptor analogue is so low that it remains undetected by SPR. The weak binding strength for α(2,3)-fetuin also accounts for the big affinity difference between the two linkages (~ 200-fold for Chita/1/03, ~ 3,500-fold for Christchurch/28/03). The Fujian-like viruses therefore feature a large shift in specificity towards the α(2,6)-linkage. However, since the specificity index only describes a relative affinity, it would be wrong to conclude that these viruses bind α(2,6)-fetuin more strongly than the earlier viruses. Indeed, none of the Fujian-like viruses binds this linkage more strongly than the viruses of 1994-1999. Whereas Christchurch/28/03, United Kingdom/1861/03 and Chita/1/03 bind α(2,6)-fetuin with similar affinity to the early viruses, Kumamoto/102/02 and Wyoming/3/03 display a considerably reduced binding strength for this linkage.

Further differences in the recognition of α(2,3)- and α(2,6)-fetuin are revealed between the viruses. Whereas the earlier viruses do not differ greatly in affinity for the receptor analogues, the Fujian-like viruses show substantial variation in recognition of both α(2,3) -and α(2,6)-fetuin. It should be noted that the "dual"-linkage specificity of Johannesburg/33/94 is mainly a result of a decreased affinity of this virus for α(2,6)-fetuin compared to X31. In contrast, the lack of linkage-discrimination for Panama/2007/99 appears to be due to an increased affinity for α(2,3)-fetuin compared to X31.

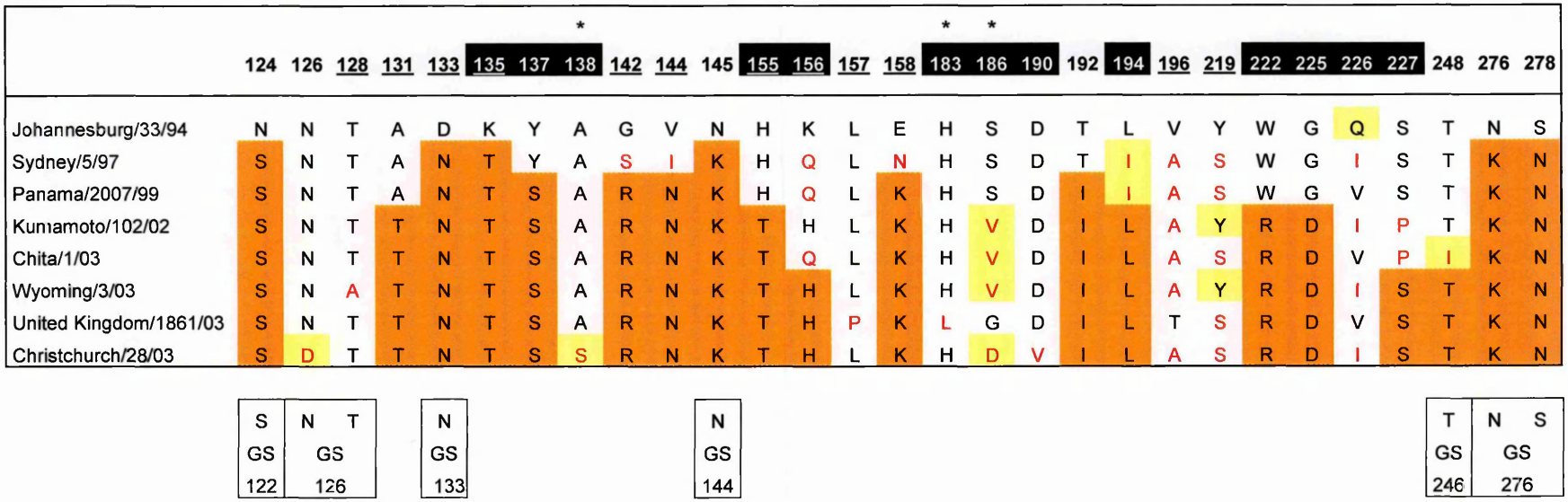
4.2.3 Location of Amino Acid Changes in HA

Since the mutation rate of the viral RNA polymerase is high (Parvin et al., 1986),

propagation of influenza virus in host cells can lead to amino acid changes. In order to compare the HA_1 sequence for the viruses used in the SPR study, it was therefore necessary to sequence this region of the HA gene upon virus isolation from eggs (*2.2.1.5 DNA Sequencing, p.77*) (see Appendix 4 for sequence alignment, p.264). An alignment of HA_1 residues, at which the viruses used in this study differ, is shown in Table 6. The amino acids were classified according to their potential to affect receptor-binding properties based on their location in the HA structure (based on the X31 HA structure). Residues considered to potentially affect the interaction with cellular receptors are 1) located within 20 Å of the RBS with side-chains pointing towards it, 2) located further away from the RBS but in a position to affect interaction with receptor analogues of neighbouring HA monomers and 3) residues which are part of a glycosylation sequon (see Appendix 5 for carbohydrate attachment sites, p.266). The remaining residues comprise amino acids located elsewhere in HA.

Figure 33 shows the location of these residues in the HA structure. As can be seen, the majority of the changes cluster around the RBS, supporting that this area is under positive selection by immune surveillance (Wiley et al., 1981; Cox and Bender, 1995; Fitch et al., 1997; Bush et al., 1999b). Importantly, a number of substitutions have occurred at residues that are part of the receptor-binding pocket itself, shaded in black in Table 6 and coloured in pink in Figure 33. The majority of the residues might be involved in antibody-recognition based on their surface-exposed side chains. Of these, the residues underlined have been directly implicated to be part of an epitope, because substitutions at these positions have previously been selected with mAb (Wiley et al., 1981; Underwood et al., 1987; Smith et al., 1991; Temoltzin-Palacios and Thomas, 1994; Patera et al., 1995).

Residue 226, considered a major determinant for linkage specificity, was shown to change from Leu → Ile → Val from 1992-1998 (Lindstrom et al., 1996; Mori et al., 1999).



	124	126	128	131	133	135	137	138*	142	144	145	155	156	157	158	183*	186*	190	192	194	196	219	222	225	226	227	248	276	278
Johannesburg/33/94	N	N	T	A	D	K	Y	A	G	V	N	H	K	L	E	H	S	D	T	L	V	Y	W	G	Q	S	T	N	S
Sydney/5/97	S	N	T	A	N	T	Y	A	S	I	K	H	Q	L	N	H	S	D	T	I	A	S	W	G	I	S	T	K	N
Panama/2007/99	S	N	T	A	N	T	S	A	R	N	K	H	Q	L	K	H	S	D	I	I	A	S	W	G	V	S	T	K	N
Kumamoto/102/02	S	N	T	T	N	T	S	A	R	N	K	T	H	L	K	H	V	D	I	L	A	Y	R	D	I	P	T	K	N
Chita/1/03	S	N	T	T	N	T	S	A	R	N	K	T	Q	L	K	H	V	D	I	L	A	S	R	D	V	P	I	K	N
Wyoming/3/03	S	N	A	T	N	T	S	A	R	N	K	T	H	L	K	H	V	D	I	L	A	Y	R	D	I	S	T	K	N
United Kingdom/1861/03	S	N	T	T	N	T	S	A	R	N	K	T	H	P	K	L	G	D	I	L	T	S	R	D	V	S	T	K	N
Christchurch/28/03	S	D	T	T	N	T	S	S	R	N	K	T	H	L	K	H	D	V	I	L	A	S	R	D	I	S	T	K	N

S	N	T	N	N	T	N	S
GS	GS		GS	GS		GS	GS
122	126		133	144		246	276

Table 6 Substitutions at H3 HA_1 residues

a) Substitutions at H3 HA_1 residues with the potential to affect receptor-binding properties Classification of residues into this group is based on 1) location within 20 Å of the RBS with side-chains pointing towards it, 2) location in a position to affect interaction with receptor analogues bound to neighbouring HA monomers, 3) being part of a glycosylation site (see Appendix 1 for amino acid one-letter code, p.254). shaded in black = located in the RBS, underlined = substitutions at residues previously selected by mAbs, shaded in yellow = likely to be a result of egg-adaptation, asterix = amino acid side-chain is "in" (0 $Å^2$ surface area exposed to water molecule with radius = 1.4 Å), shaded in orange = residue does not change anymore, colour change for amino acids indicates a substitution compared to previous virus, boxes indicate that a glycosylation site (GS) is present at residue indicated below if residues above are as present.

	21	25	50	57	62	75	83	105	121	172	197	202 *	233 *	262
Johannesburg/33/94	P	L	R	R	K	H	E	Y	I	G	R	V	H	N
Sydney/5/97	P	L	R	R	E	H	E	Y	N	D	Q	V	H	S
Panama/2007/99	S	L	R	Q	E	H	E	Y	N	E	Q	V	Y	S
Kumamoto/102/02	P	I	G	Q	E	Q	K	Y	N	E	Q	I	Y	S
Chita/1/03	P	I	G	Q	E	Q	K	Y	N	E	Q	I	Y	S
Wyoming/3/03	P	I	G	Q	E	Q	K	Y	N	E	Q	I	Y	S
United Kingdom/1861/03	P	I	G	Q	E	Q	K	Y	N	E	Q	I	Y	S
Christchurch/28/03	P	I	G	Q	E	Q	K	H	N	E	Q	I	Y	S

Table 6 (continued) Substitutions at H3 HA_1 residues

b) Substitutions at H3 HA_1 residues less likely to affect receptor-binding properties This group contains residues that are located elsewhere in HA than those in Table 6a). underlined = substitutions at residues previously selected by mAbs, asterix = amino acid side-chain is "in" (0 $Å^2$ surface area exposed to water molecule with radius = 1.4 Å), shaded in orange = residue does not change anymore, colour change for amino acids indicates a substitution compared to previous virus (see Appendix 1 for amino acid one-letter code, p.254)

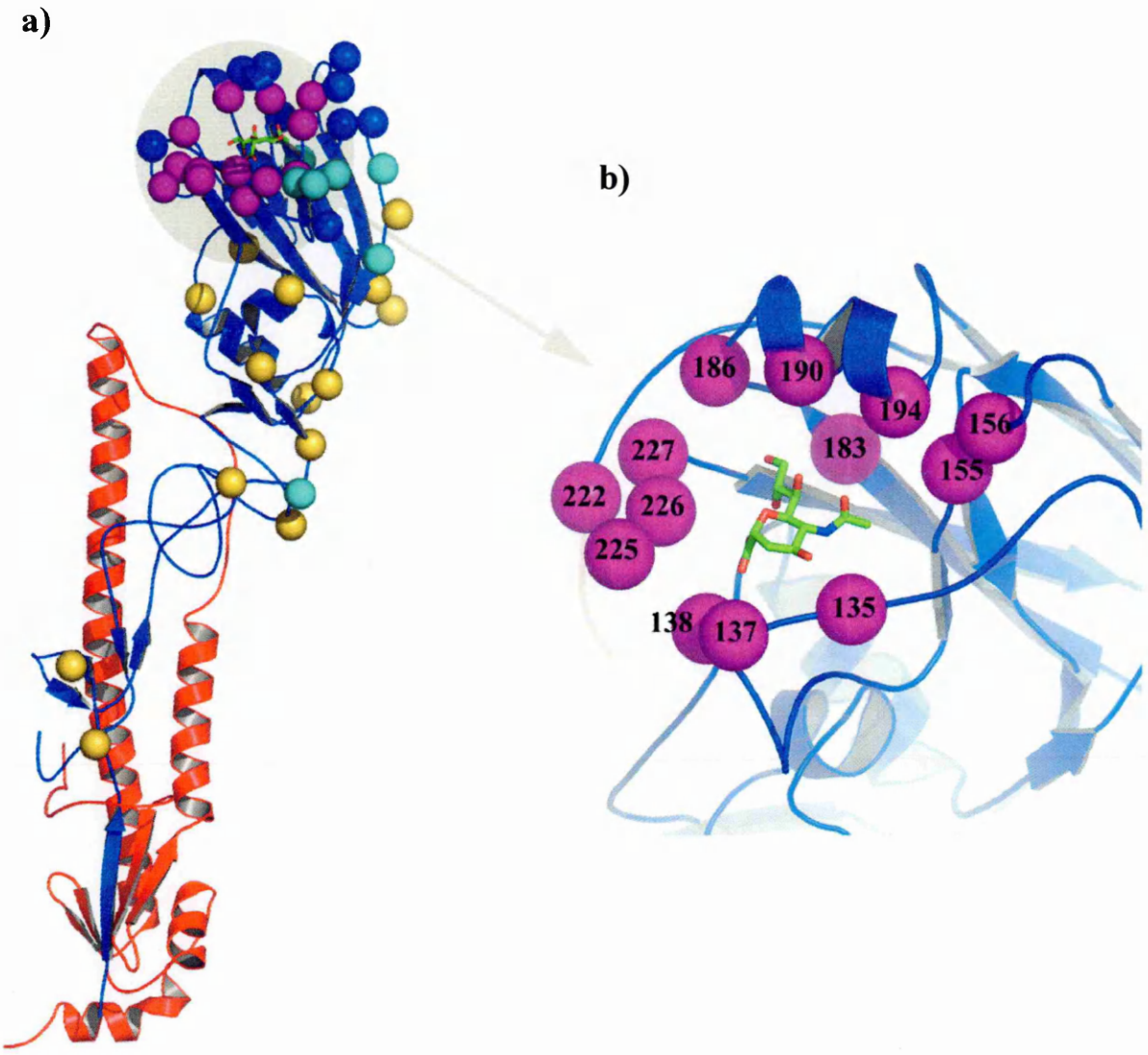


Figure 33 Schematic diagram showing residues containing changes in HA_1 for H3 viruses used in this study

a) HA monomer showing the location of all the changes. The different colours represent the classification of the residues. Circles in pink are residues located in the receptor-binding site (residues shaded in black in Table 6a). Circles in blue are residues located within 20 Å of the receptor-binding site or residues that are in a position to affect interaction with receptor analogues bound to neighbouring monomers. Circles in cyan are carbohydrate attachment sites. Circles in yellow are residues located elsewhere in HA_1.**b)** Close-up of the receptor-binding site and the residues at which changes are observed. Bound Neu5Ac is coloured in green.

Since these amino acids are non-polar, they all fulfil the criterion for binding to α(2,6)-linked Neu5Ac (see Figure 7, p.42). All the viruses used in this study contained either Ile or Val, indicating that Val226 has not become fixed in HA evolution. The only exception to this is Johannesburg/33/94, which contains the avian-typical Gln at this position. As described below, this is likely to be the result of growth of this virus in eggs.

4.2.4 Assessment of Egg-Adaptation Mutations

As stated in *4.1.3 Host Cell-Mediated Variation, p.134*, the viruses used in this study might contain egg-adaptation mutations, which are likely to affect receptor-binding properties. Comparison of HA_1 residues that had previously been associated with egg-adaptation (see Table 3) suggests that the following viruses might contain substitutions implicated in this process: Chita/1/03 (Val186, Ile248), Christchurch/28/03 (Asp126, Ser138, Asp186,), Panama/2007/99 (Ile194), Sydney/5/97 (Ile194), Johannesburg/33/94 (Gln226), Kumamoto/102/02 (Val186, Tyr219) and Wyoming/3/03 (Val186, Tyr219). These residues are shaded in yellow in Table 6. How these particular potential egg-adaptation mutations would affect the interaction with α(2,3)- and α(2,6)-fetuin is uncertain, since for none of these pairwise comparison of receptor-binding properties for egg-adapted and MDCK-grown viruses has been performed (see Table 3). However, since residue 226 has been shown to be a major determinant for linkage specificity for the H3 subtype (see *1.7.4.2 Correlation between Receptor-Linkage Specificity and Host of Origin, p.39*), the amino acid at this residue is expected to influence receptor-binding properties. An increase in affinity for the α(2,3)-linkage with or without concomitant decrease in binding strength for the α(2,6)-linkage has been reported for egg-adapted variants containing the Leu226Gln mutation, although they differed at additional residues as well (Ito et al., 1997b). The "dual"-specificity of Johannesburg/33/94 is therefore likely to be

the result of a decreased affinity for the α(2,6)-linkage due to an egg-adaptation substitution at residue 226. Notably, many viruses isolated from 1992-1994 were shown to contain the avian-typical Gln226, which have also been suggested to be the result of egg-adaptation (Lindstrom et al., 1996). Differences at other residues associated with egg-adaptation (137, 145, 155, 156, 158, 159 and 186) have also been identified for the viruses used in this study. However, since changes at these residues have been shown to be characteristic for the emergence of antigenic/genetic variants in the course of HA evolution (WIC, London, in-house sequencing), these are less likely to be the result of host cell-mediated variation.

4.3 Receptor-Binding Properties of H1 Subtype Viruses

4.3.1 Antigenic and Phylogenetic Relationships

The receptor-binding properties of a panel of H1 subtype viruses were also investigated by SPR. These viruses were introduced into the human population in 1918, causing the Spanish pandemic, circulated until 1957 and were then replaced by the emerging H2 subtype viruses. However, they reappeared in 1977 and since then continue to co-circulate with H3 subtype viruses. Therefore, viruses isolated from both epidemic periods were tested by SPR, starting with one of the first human viruses isolated, A/Puerto Rico/8/34. In addition to the human viruses, a virus of swine origin, Sw/Iowa/15/30, was included in the study. This was the first H1N1 virus isolated from a mammal, which emerged in pigs around the same time as in humans (1918). Human and Sw/Iowa/15/30-like viruses have been shown to share a common avian-like ancestor but have evolved into distinct lineages, human H1N1 and "classical" swine H1N1 viruses (Gorman et al., 1991). The pig isolate was included in the study in order to determine to what extent the receptor-binding properties have diverged upon evolution in the two different hosts. Furthermore, in

contrast to all the other viruses being of the H1N1 subtype, the study also included an H1N2 virus. These viruses appeared in 2001 and arose by reassortment between co-circulating H3N2 and H1N1 viruses (WHO, 2002c; WHO, 2002b). It has been shown that the H1N2 viruses contain an HA closely related antigenically and genetically to contemporary A/New Caledonia/20/99 (H1N1)-like viruses, while the NA and the other six gene segments are closely related to A/Moscow/10/99 (H3N2)-like viruses (Gregory et al., 2002). Since their introduction the H1N2 viruses have continued to circulate as a separate genetic lineage in various parts of the world together with H1N1 and H3N2 viruses.

The antigenic relationships of the viruses from both epidemic periods used in this study are shown in Tables 7 and 8. All the viruses isolated from 1934-1957 represent distinct antigenic variants as indicated by the lack of cross-reactivity with post-infection ferret sera, except for Finland/9/57 and Denver/1/57, which are closely related. Although isolated early in the second epidemic period, Brazil/11/78 was included in the HI tests with viruses from the first period. Soon after the re-emergence of the H1N1 viruses in 1977, it was noted that these viruses were related antigenically and genetically more closely to viruses isolated in 1949-50 than to strains which circulated before or after that period (Kendal et al., 1978; Nakajima et al., 1978). The data presented in Table 7 confirm the antigenic relatedness between Brazil/11/78 and Fort Warren/1/50. The antigenic properties of the viruses used in this study of 1977-2003 are shown in Table 8. Except for Taiwan/1/86 and Bayern/7/95, displaying antigenic similarity, the viruses from 1977-1999 are antigenically distinct. However, the data also shows that all later viruses are New Caledonia/20/99-like, including the H1N2 virus Egypt/96/02. This is in agreement with the reported slow antigenic evolution between 1986-1995 and 1999-2003 (Hay et al., 2001). Compared to the H3 viruses, antigenic evolution has been shown to be slower for H1

	Haemagglutination inhibition titre					
	Post -infection ferret sera					
Virus	PR/8/34	FM/1/47	FW/1/50	FLW/1/52	Denver/1/57	Brazil/11/78
Puerto Rico/8/34	**2560**	<	<	40	40	<
Fort Monmouth/1/47	<	**640**	160	40	<	320
Fort Warren/1/50	<	40	**320**	<	<	320
Fort Leonard Wood/1/52	<	<	40	**2560**	40	<
Denver/1/57	<	<	<	<	**1280**	<
Finland/9/57	<	<	<	<	2560	<
Brazil/11/78	<	80	320	40	<	**640**

Table 7 Antigenic analysis of H1N1 viruses 1934-1978 Haemagglutination inhibition (HI) reactions of post-infection ferret sera with influenza H1N1 viruses. HI titres represent the reciprocal of the highest dilution of ferret sera inhibiting 8 haemagglutination units of virus. < represents titres less than 40, homologous titres are in bold. No antiserum was available for Finland/9/57.

	Haemagglutination inhibition titre								
	Post-infection ferret sera								
Virus	Bra/11/78	Chile/1/83	Taiw/1/86	Bay/7/95	Beij/262/95	NC/20/99	Mad/57794/00	Chile/888/02	Egy/96/02 (H1N2)
Brazil/11/78	**640**	<	<	<	<	<	<	<	<
Chile/1/83	40	**640**	<	<	<	<	<	<	<
Taiwan/1/86	<	40	**640**	640	<	<	<	<	<
Bayern/7/95	<	<	320	**1280**	80	80	80	40	40
Beijing/262/95	<	<	<	40	**1280**	640	640	320	640
NewCaledonia/20/99	<	<	<	<	160	**1280**	1280	1280	1280
Madagascar/57794/00	<	<	<	<	160	1280	**1280**	1280	640
Chile/8885/02	<	<	<	40	80	640	1280	**1280**	640
Bucharest/955/03	<	<	<	40	160	320	320	640	320
Prague/9/03	<	<	<	<	40	640	320	640	320
Egypt/96/02 (H1N2)	<	<	<	<	160	640	1280	640	**1280**

Table 8 Antigenic analysis of H1N1 and H1N2 viruses 1978-2003 Haemagglutination inhibition (HI) reactions of post-infection ferret sera with influenza H1N1 and H1N2 viruses. HI titres represent the reciprocal of the highest dilution of ferret sera inhibiting 8 haemagglutination units of virus. < represents titres less than 40, homologous titres are in bold. No antiserum was available for Bucharest/955/03 and Prague/9/03.

viruses isolated from the second epidemic period. This is reflected by only 10 changes in the recommended vaccine composition from 1977-2001 compared to 19 for the H3 viruses from 1972-2001 (Hay et al., 2001).

The phylogenetic relationships between the viruses from both epidemic periods are shown in Figure 34. The close genetic relationship between Brazil/11/78 and Fort Warren/1/50 is in agreement with previous reports (Nakajima et al., 1978). This finding also shows that, in contrast to H3 viruses, which evolve as a single lineage, H1 antigenic variants do not always evolve directly from the preceding circulating strains. Similar observations of divergent evolution can also be made for antigenic variants that emerged in 1995 (represented by Beijing/262/95). The HA genes of antigenically distinct Beijing/262/95-like and Bayern/77/95-like viruses had already diverged in early 1990 into two distinct phylogenetic lineages. These viruses co-circulated until Bayern/262/95-like viruses were replaced in 2001 by New Caledonia/20/99-like viruses (Hay et al., 2001). Although the currently circulating viruses are all antigenically similar to New Caledonia/20/99, Figure 34 shows that they can be classified into 3 different phylogenetic lineages. H1N1 variant group 1 is represented by New Caledonia/20/99, Bucharest/955/03 and Prague/9/03, whereas Madagascar/57794/00 and Chile/8885/02 are classified into H1N1 variant group 2. Finally, Egypt/96/02 is an example of the reassortant group H1N2 (group 3), with its HA closely related to H1N1 variant group 2.

4.3.2 Affinity and Specificity Studies by SPR

The receptor-binding properties of these human H1 viruses and the pig isolate, Sw/Iowa/15/30, were tested by SPR as described for the H3 subtype viruses. Dk/Alberta/35/76 was included in the assay as an example of a bird isolate. As for the H3 subtype, all the viruses were propagated in eggs for reasons stated earlier. The rate

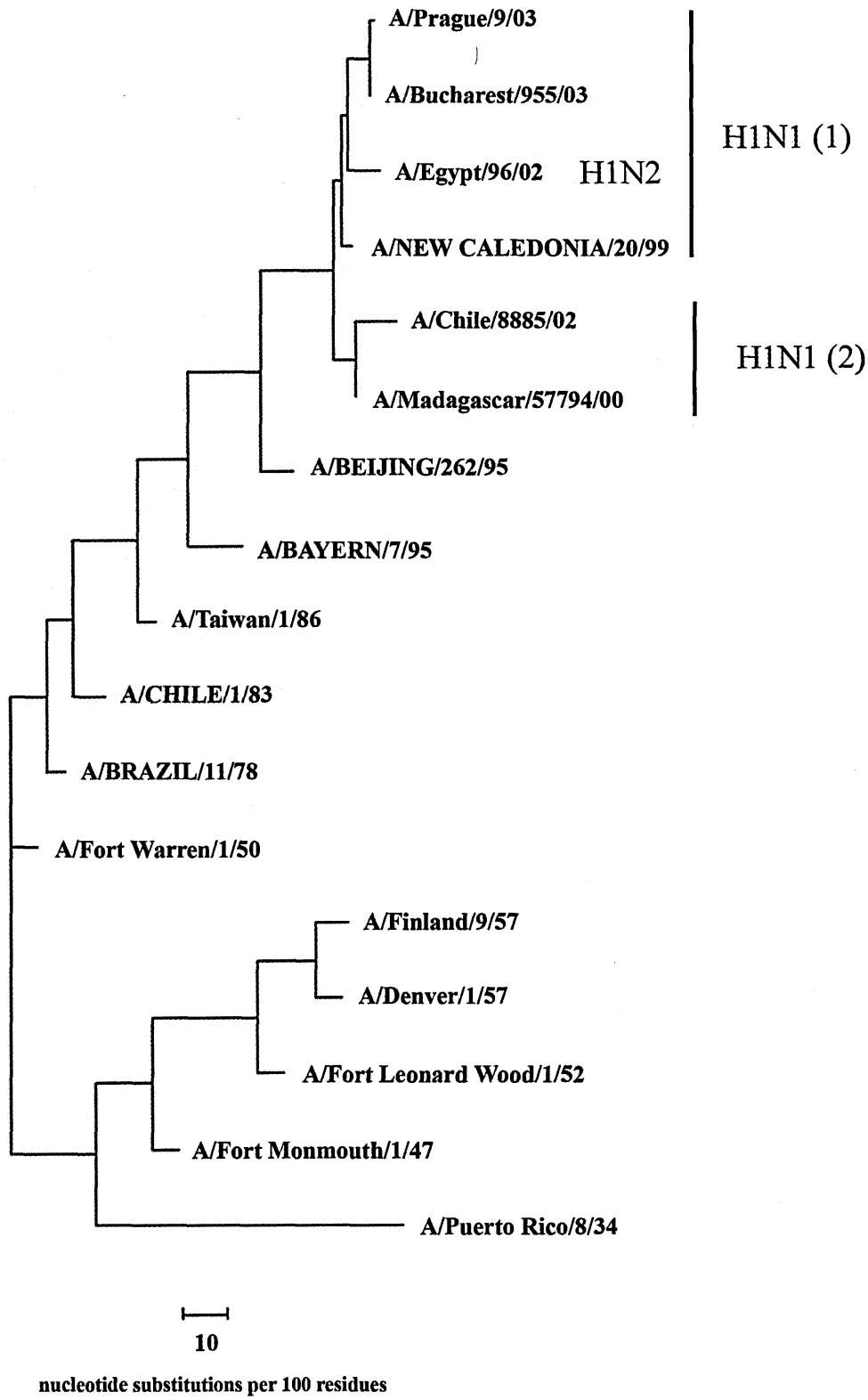


Figure 34 Phylogenetic comparison of H1 HA genes
The sequences of nucleotides 74-995 encoding the HA_1 domain were compared using *Phylogenetic Analysis Using Parsimony*, version 4.0 (Swofford and Olsen, 1990). The lengths of the horizontal lines are proportional to the number of nucleotide differences, as indicated by the bar. Vaccine strains are indicated in capital letters. The two distinguishable phylogenetic lineages of viruses 1999-2003 are indicated in blue and the reassortant virus in red.

constants and affinities for α(2,3)- and α(2,6)-fetuin are shown in Table 9 and the affinities displayed in log-scale in Figure 35. Dk/Alberta/35/76 is characterised by a clear α(2,3)-linkage specificity, similar to the H3 bird isolate Dk/Ukraine/1/63. However, in contrast to the avian H3 virus, the affinity of this virus for the non-preferred α(2,6)-linkage was so low that no binding to the respective receptor analogue was detected. The human viruses and the pig isolate can be grouped into 3 different classes with regard to their linkage specificity. Class 1 is characterised by the preferential recognition of α(2,3)-fetuin, represented mostly by strains from the first epidemic period (Puerto Rico/8/34, Fort Leonard Wood/52, Denver/1/57 and Finland/9/57). In contrast, only two of the post-1977 viruses are classified as α(2,3)-linkage specific (Taiwan/1/86 and Bayern/7/95). Class 2 viruses appear not to distinguish significantly between α(2,3)- and α(2,6)-fetuin and are therefore classified as "dual"-linkage specific. This class is represented by Fort Monmouth/1/47, Fort Warren/1/50, Brazil/11/78, Chile/1/83, Beijing/262/95 and the pig isolate Sw/Iowa/15/30. A third class of viruses preferentially recognise α(2,6)-fetuin. Whereas none of the viruses from the first epidemic era appears to be clearly α(2,6)-specific, all the viruses isolated since 1999 including the H1N2 virus Egypt/96/02 are classified into this group.

4.3.3 Location of Amino Acid Changes in HA

As described for the H3 viruses, the H1 viruses were sequenced in order to reveal the changes in HA_1 amino acid sequence (see Appendix 6 for alignments, p.267). Classification of these according to their potential to affect receptor-binding properties was based on the structure of Sw/Iowa/15/30 HA and is shown in Table 10. The same criterion was used as for the H3 viruses, with residues likely to affect interaction with cellular receptors being 1) located within 20 Å of the RBS with side-chains pointing towards it, 2)

Viruses	α(2,6)-fetuin			α(2,3)-fetuin			Specificity Index
	10^{-6} x k_a ($M^{-1}s^{-1}$)	10^{6} x k_d (s^{-1})	10^{14} x K_D (M)	10^{-6} x k_a ($M^{-1}s^{-1}$)	10^{6} x k_d (s^{-1})	10^{14} x K_D (M)	
Puerto Rico/8/34	8 ± 0.8	50 ± 20	**610 ± 250**	20 ± 2	15 ± 3	**74 ± 16**	0.1
Sw/Iowa/15/30	12 ± 1	8 ± 4	**65 ± 33**	19 ± 1	5 ± 2	**27 ± 11**	0.4
Fort Monmouth/1/47	17 ± 1	4 ± 1.4	**24 ± 8**	23 ± 2	12 ± 4	**51 ± 18**	2.1
Fort Warren/1/50	17 ± 1	2 ± 1	**12 ± 6**	25 ± 1	1 ± 0.5	**5 ± 2**	0.4
Fort Leonard Wood/1/52	2 ± 0.6	60 ± 15	**2,400 ± 860**	21 ± 2	8 ± 4	**38 ± 19**	0.02
Denver/1/57	3 ± 0.3	80 ± 25	**2,900 ± 960**	23 ± 2	4 ± 1.5	**18 ± 7**	0.006
Finland/9/57	3 ± 0.9	90 ± 25	**2,700 ± 1000**	26 ± 1	4 ± 1	**13 ± 4**	0.005
Brazil/11/78	10 ± 1	6 ± 2	**59 ± 20**	15 ± 1	15 ± 5	**98 ± 33**	1.7
Chile1/83	9 ± 0.9	4 ± 1.5	**38 ± 17**	13 ± 1	14 ± 5	**110 ± 40**	2.9
Taiwan/1/86	16 ± 1	35 ± 10	**220 ± 65**	30 ± 2	8 ± 2	**27 ± 7**	0.1
Bayern/7/95	16 ± 1	25 ± 8	**160 ± 50**	26 ± 2	10 ± 4	**39 ± 16**	0.2
Beijing/262/95	14 ± 1	25 ± 10	**180 ± 71**	21 ± 1	50 ± 15	**240 ± 71**	1.3
New Caledonia/20/99	15 ± 1	2 ± 1.1	**15 ± 7**	18 ± 1	40 ± 15	**220 ± 83**	15
Madagascar/57794/00	24 ± 2	6 ± 2	**25 ± 8**	26 ± 2	60 ± 15	**230 ± 60**	9.2
Chile/8885/02	14 ± 1	4 ± 1.5	**29 ± 10**	27 ± 2	57 ± 15	**210 ± 57**	7.2
Bucharest/955/03	34 ± 2	5 ± 2	**15 ± 6**	29 ± 2	54 ± 21	**190 ± 74**	13
Prague/9/03	30 ± 2	5 ± 2	**17 ± 7**	25 ± 2	53 ± 20	**210 ± 85**	12
Egypt/96/02 (H1N2)	29 ± 2	2 ± 0.6	**6 ± 2.1**	2 ± 0.5	220 ± 80	**14,300 ± 6700**	2400
Dk/Alberta/35/76	<	>	>	18 ± 1	1 ± 0.5	**7 ± 2.9**	<

Table 9 Kinetic rate constants and affinities for the interaction of H1N1 and H1N2 viruses with α(2,6)- and α(2,3)-fetuin The association rate constants (k_a) and dissociation rate constants (k_d) were determined by SPR. Virus at four different concentrations was injected over the BIAcore sensor chip containing immobilised derivatised fetuins. Kinetic rate constants were determined as described in *Chapter 3* and the affinity expressed as k_d/k_a (K_D). High K_D values indicate low affinity. < and > indicate that values were below detection limit. The specificity index (SI) is K_D (α2,3)/K_D (α2,6) and is a measure for the linkage preference of a virus, with < 1 = preference for α(2,3)- and >1 = preference for α(2,6)-fetuin. α(2,6)-specific viruses are indicated in red, α(2,3)-specific viruses in blue and "dual"-specific viruses in black.

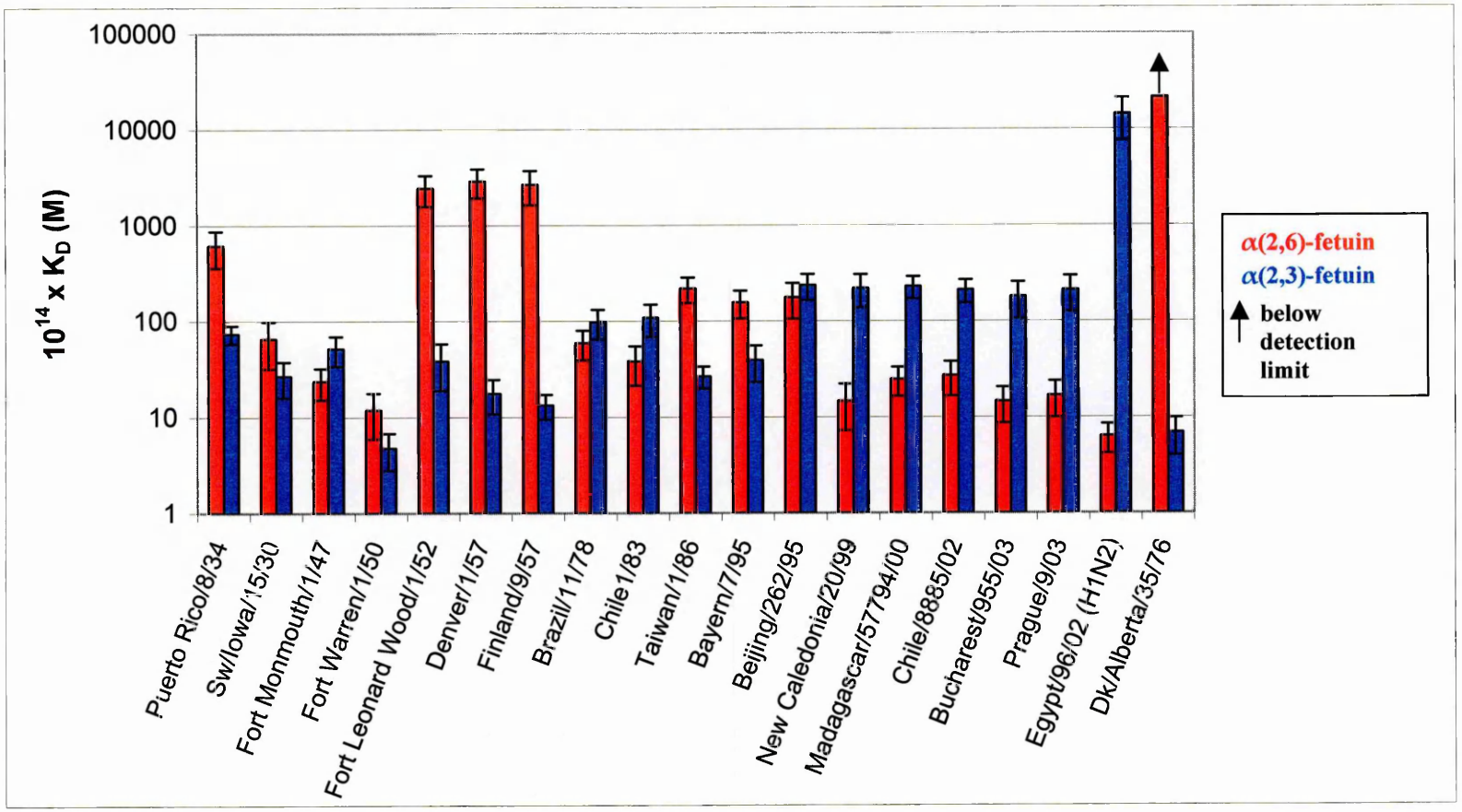


Figure 35 Affinities of H1N1 and H1N2 viruses for α(2,6) and α(2,3)-fetuin The calculated K_D values shown in Table 9 are displayed in log-scale. High values indicate low affinity. Black arrows indicate that the affinities were too low to be detected by SPR.

Table 10 Substitutions at H1 HA_1 residues

a) Substitutions at H1 HA_1 residues with the potential to affect receptor-binding properties Classification of residues into this group is based on 1) location within 20 Å of the RBS with side-chains pointing towards it, 2) location in a position to affect interaction with receptor analogues bound to neighbouring HA monomers, 3) being part of a glycosylation site (see Appendix 1 for amino acid one-letter code, p.254). shaded in black = located in the RBS, underlined = substitutions at residues previously selected by mAbs, shaded in yellow = likely to be a result of egg-adaptation, asterix = amino acid side-chain is "in" (0 $Å^2$ surface area exposed to water molecule with radius = 1.4 Å), shaded in orange = residue does not change anymore, colour change for amino acids indicates a substitution compared to previous virus, boxes indicate that a glycosylation site (GS) is present at residue indicated below if residues above are as present.

Virus abbreviations:		
	PR8	Puerto Rico/8/34
	FM47	Fort Monmouth/1/47
	FW50	Fort Warren/1/50
	FLW52	Fort Leonard Wood/1/52
	Denv57	Denver/1/57
	Fin57	Finland/9/57
	Braz78	Brazil/11/78
	Chil83	Chile/1/83
	Taiw86	Taiwan/1/86
	Bay95	Bayern/7/95
	Beij95	Beijing/262/95
	Cal99	New Caledonia/20/99
	Mad00	Madagascar/57794/00
	Chil02	Chile/8885/02
	Buch03	Bucharest/955/03
	Prag03	Prague/9/03
	Eg02	Egypt/96/02

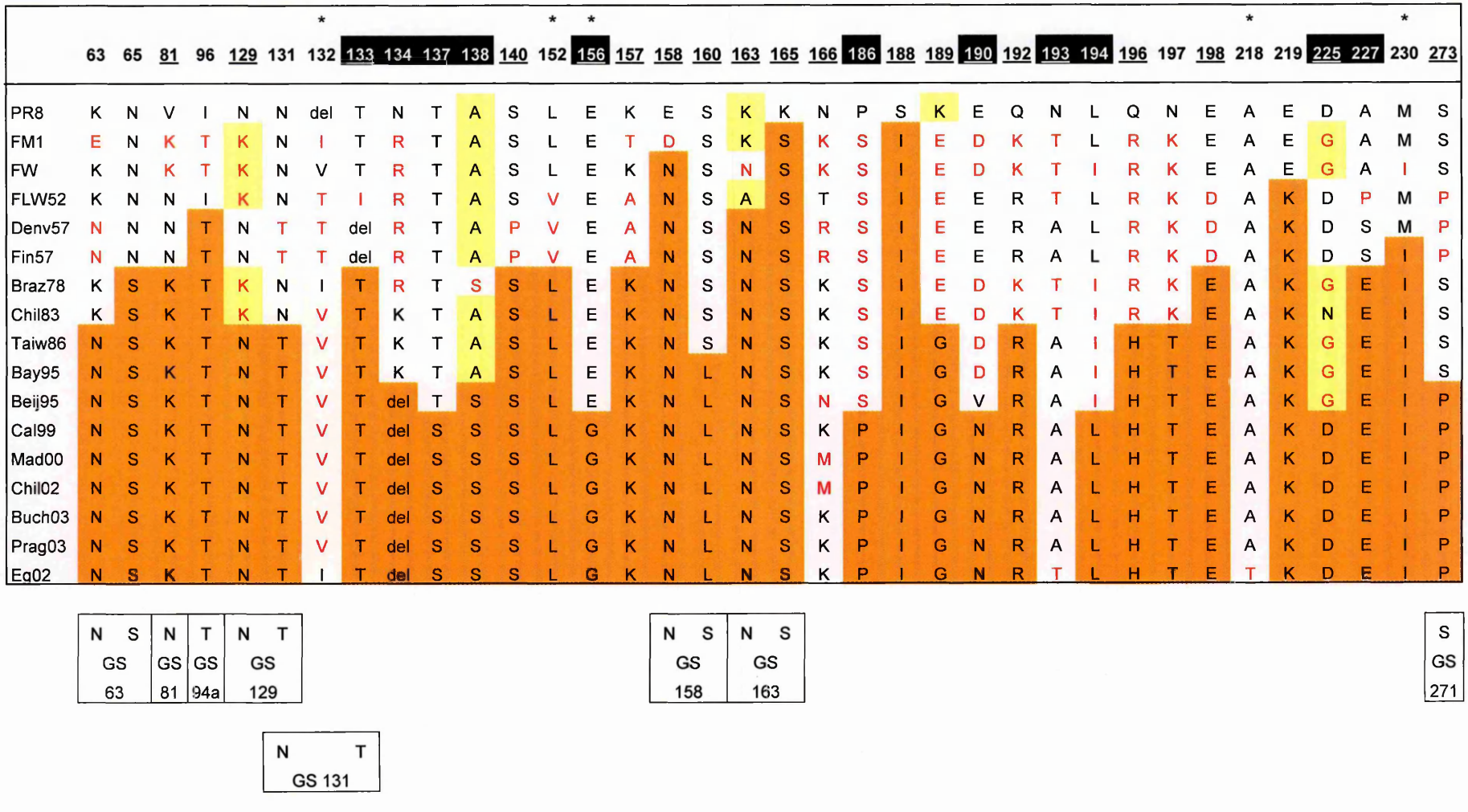
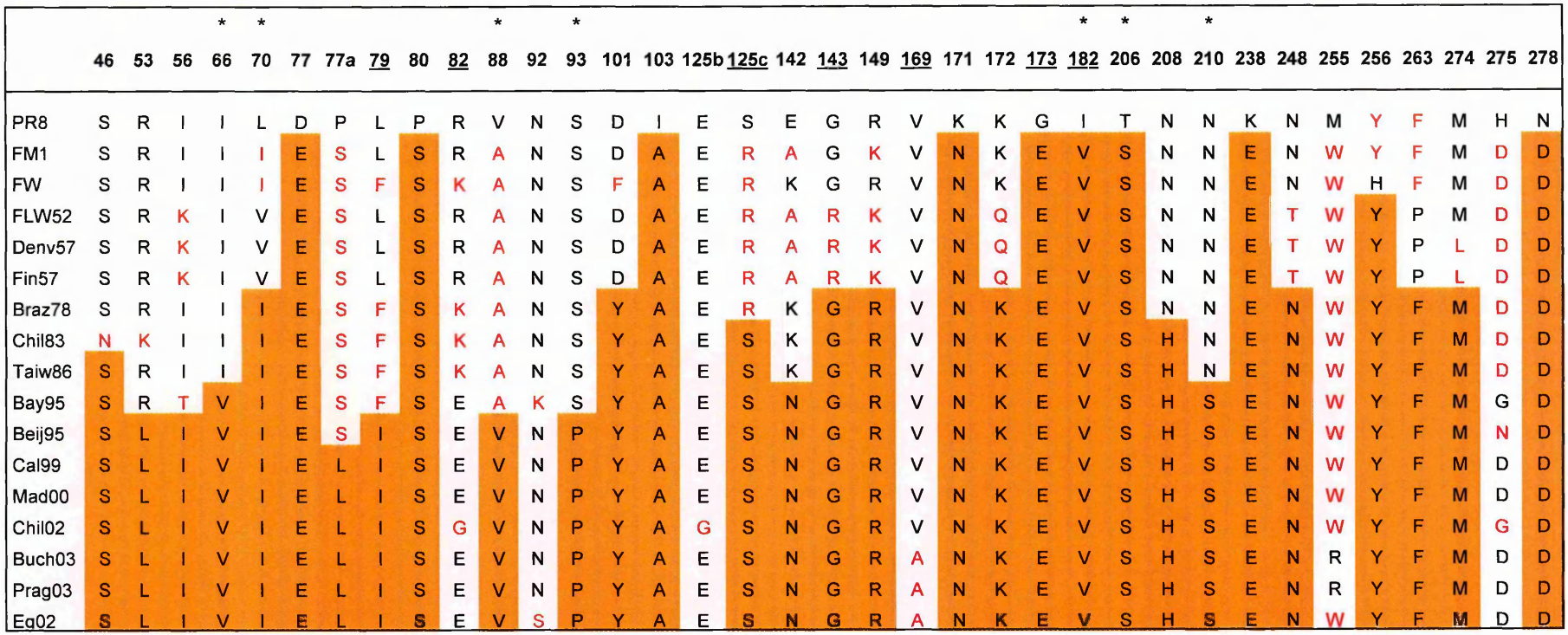

63 65 81 96 129 131 132 133 134 137 138 140 152 156 157 158 160 163 165 166 186 188 189 190 192 193 194 196 197 198 218 219 225 227 230 273
PR8 K N V I N N del T N T A S L E K E S K K N P S K E Q N L Q N E A E D A M S
FM1 E N K T K N I T R T A S L E T D S K S K S I E D K T L R K E A E G A M S
FW K N K T K N V T R T A S L E K N S N S K S I E D K T I R K E A E G A I S
FLW52 K N N I K N T I R T A S V E A N S A S T S I E E R T L R K D A K D P M P
Denv57 N N N T N T T del R T A P V E A N S N S R S I E E R A L R K D A K D S M P
Fin57 N N N T N T T del R T A P V E A N S N S R S I E E R A L R K D A K D S I P
Braz78 K S K T K N I T R T S S L E K N S N S K S I E D K T I R K E A K G E I S
Chil83 K S K T K N V T K T A S L E K N S N S K S I E D K T I R K E A K N E I S
Taiw86 N S K T N T V T K T A S L E K N S N S K S I G D R A I H T E A K G E I S
Bay95 N S K T N T V T K T A S L E K N L N S K S I G D R A I H T E A K G E I S
Beij95 N S K T N T V T del T S S L E K N L N S N S I G V R A I H T E A K G E I P
Cal99 N S K T N T V T del S S S L G K N L N S K P I G N R A L H T E A K D E I P
Mad00 N S K T N T V T del S S S L G K N L N S M P I G N R A L H T E A K D E I P
Chil02 N S K T N T V T del S S S L G K N L N S M P I G N R A L H T E A K D E I P
Buch03 N S K T N T V T del S S S L G K N L N S K P I G N R A L H T E A K D E I P
Prag03 N S K T N T V T del S S S L G K N L N S K P I G N R A L H T E A K D E I P
Eq02 N S K T N T I T del S S S L G K N L N S K P I G N R T L H T E T K D E I P
N S N T N T
GS GS GS GS
63 81 94a 129
N T
GS 131
N S N S
GS GS
158 163
S
GS
271

Table 10a



	46	53	56	66*	70*	77	77a	79	80	82	88*	92	93*	101	103	125b	125c	142	143	149	169	171	172	173	182*	206*	208	210*	238	248	255	256	263	274	275	278
PR8	S	R	I	I	L	D	P	L	P	R	V	N	S	D	I	E	S	E	G	R	V	K	K	G	I	T	N	N	K	N	M	Y	F	M	H	N
FM1	S	R	I	I	I	E	S	L	S	R	A	N	S	D	A	E	R	A	G	K	V	N	K	E	V	S	N	N	E	N	W	Y	F	M	D	D
FW	S	R	I	I	I	E	S	F	S	K	A	N	S	F	A	E	R	K	G	R	V	N	K	E	V	S	N	N	E	N	W	H	F	M	D	D
FLW52	S	R	K	I	V	E	S	L	S	R	A	N	S	D	A	E	R	A	R	K	V	N	Q	E	V	S	N	N	E	T	W	Y	P	M	D	D
Denv57	S	R	K	I	V	E	S	L	S	R	A	N	S	D	A	E	R	A	R	K	V	N	Q	E	V	S	N	N	E	T	W	Y	P	L	D	D
Fin57	S	R	K	I	V	E	S	L	S	R	A	N	S	D	A	E	R	A	R	K	V	N	Q	E	V	S	N	N	E	T	W	Y	P	L	D	D
Braz78	S	R	I	I	I	E	S	F	S	K	A	N	S	Y	A	E	R	K	G	R	V	N	K	E	V	S	N	N	E	N	W	Y	F	M	D	D
Chil83	N	K	I	I	I	E	S	F	S	K	A	N	S	Y	A	E	S	K	G	R	V	N	K	E	V	S	H	N	E	N	W	Y	F	M	D	D
Taiw86	S	R	I	I	I	E	S	F	S	K	A	N	S	Y	A	E	S	K	G	R	V	N	K	E	V	S	H	N	E	N	W	Y	F	M	D	D
Bay95	S	R	T	V	I	E	S	F	S	E	A	K	S	Y	A	E	S	N	G	R	V	N	K	E	V	S	H	S	E	N	W	Y	F	M	G	D
Beij95	S	L	I	V	I	E	S	I	S	E	V	N	P	Y	A	E	S	N	G	R	V	N	K	E	V	S	H	S	E	N	W	Y	F	M	N	D
Cal99	S	L	I	V	I	E	L	I	S	E	V	N	P	Y	A	E	S	N	G	R	V	N	K	E	V	S	H	S	E	N	W	Y	F	M	D	D
Mad00	S	L	I	V	I	E	L	I	S	E	V	N	P	Y	A	E	S	N	G	R	V	N	K	E	V	S	H	S	E	N	W	Y	F	M	D	D
Chil02	S	L	I	V	I	E	L	I	S	G	V	N	P	Y	A	G	S	N	G	R	V	N	K	E	V	S	H	S	E	N	W	Y	F	M	G	D
Buch03	S	L	I	V	I	E	L	I	S	E	V	N	P	Y	A	E	S	N	G	R	A	N	K	E	V	S	H	S	E	N	R	Y	F	M	D	D
Prag03	S	L	I	V	I	E	L	I	S	E	V	N	P	Y	A	E	S	N	G	R	A	N	K	E	V	S	H	S	E	N	R	Y	F	M	D	D
Eq02	S	L	I	V	I	E	L	I	S	E	V	S	P	Y	A	E	S	N	G	R	A	N	K	E	V	S	H	S	E	N	W	Y	F	M	D	D

Table 10b Substitutions at H1 HA_1 residues less likely to affect receptor-binding properties

This group contains residues that are located elsewhere in HA than those in Table 10a). underlined = substitutions at residues previously selected by mAbs, asterix = amino acid side-chain is "in" (0 $Å^2$ surface area exposed to water molecule with radius = 1.4 Å), shaded in orange = residue does not change anymore, colour change for amino acids indicates a substitution compared to previous virus (see Appendix 1 for amino acid one-letter code, p.254)

	* 279	285	* 294	* 297	310	312
PR8	T	L	Y	I	R	A
FM1	T	Q	F	I	K	T
FW	T	Q	F	I	R	T
FLW52	T	Q	F	I	R	T
Denv57	T	Q	F	I	R	T
Fin57	T	Q	F	I	R	T
Braz78	T	Q	F	V	R	T
Chil83	A	Q	F	V	R	T
Taiw86	A	Q	F	V	R	T
Bay95	A	Q	F	V	R	T
Beij95	A	Q	F	V	R	T
Cal99	A	Q	F	V	R	A
Mad00	A	Q	F	V	R	A
Chil02	A	Q	F	V	R	A
Buch03	A	Q	F	V	R	A
Prag03	A	Q	F	V	R	A
Eg02	A	Q	F	V	R	A

Table 10b (continued) Substitutions at H1 HA_1 residues less likely to affect receptor-binding properties
This group contains residues that are located elsewhere in HA than those in Table 10a). underlined = substitutions at residues previously selected by mAbs, asterix = amino acid side-chain is "in" (0 $Å^2$ surface area exposed to water molecule with radius = 1.4 Å), shaded in orange = residue does not change anymore, colour change for amino acids indicates a substitution compared to previous virus (see Appendix 1 for amino acid one-letter code, p.254)

located further away from the RBS but in a position to affect interaction with receptors of neighbouring HA monomers and 3) being part of glycosylation sequon (see Appendix 7 for carbohydrate attachment sites, p.269). Figure 36 shows the location of these residues in the structure of HA. As observed for the H3 viruses, the majority of these residues have the potential to be involved in antibody-recognition, because of their surface-exposed side-chains. Of these, the residues underlined in Table 10a have been directly implicated to be part of an epitope, because substitutions at these positions have previously been selected with mAb (Caton et al., 1982; Nakajima et al., 1983; Yates et al., 1990). A number of substitutions also involve amino acids in the RBS, shaded in black in Table 10a and pink in Figure 36. Of these, residues 138, 186, 190, 194 and 225 have been suggested to be involved in defining receptor specificity for the H1 subtype (Rogers and D'Souza, 1989; Matrosovich et al., 1997; Gambaryan et al., 1999; Mochalova et al., 2003).

4.3.4 Assessment of Egg-Adaptation Mutations

Evaluation of HA_1 amino acid sequence with regard to egg-adaptation mutations reveals that many H1 isolates of 1934-1999 contain amino acids previously associated with cultivation in eggs (see Table 3): Puerto Rico/8/34 (Ala138, Lys163, Lys189,), Fort Monmouth/1/47 (Lys129, Ala138, Lys163, Gly225), Fort Warren/1/50 (Lys129, Ala138, Gly225), Fort Leonard Wood/1/52 (Lys129, Ala138, Ala163), Denver/1/57 and Finland/9/57 (Ala138), Brazil/11/78 (Lys129, Gly225), Chile/1/83 (Lys129, Ala138, Asn225), Taiwan/1/86 (Ala138, Gly225), Bayern/7/95 (Ala138, Gly225), Beijing/262/95 (Gly225). These residues are shaded in yellow in Table 10. Changes at other residues that had previously been associated with propagation in eggs have been shown to be characteristic for the emergence of antigenic/genetic variants in the course of HA evolution (WIC, London, in-house sequencing). These are therefore less likely to be the result of

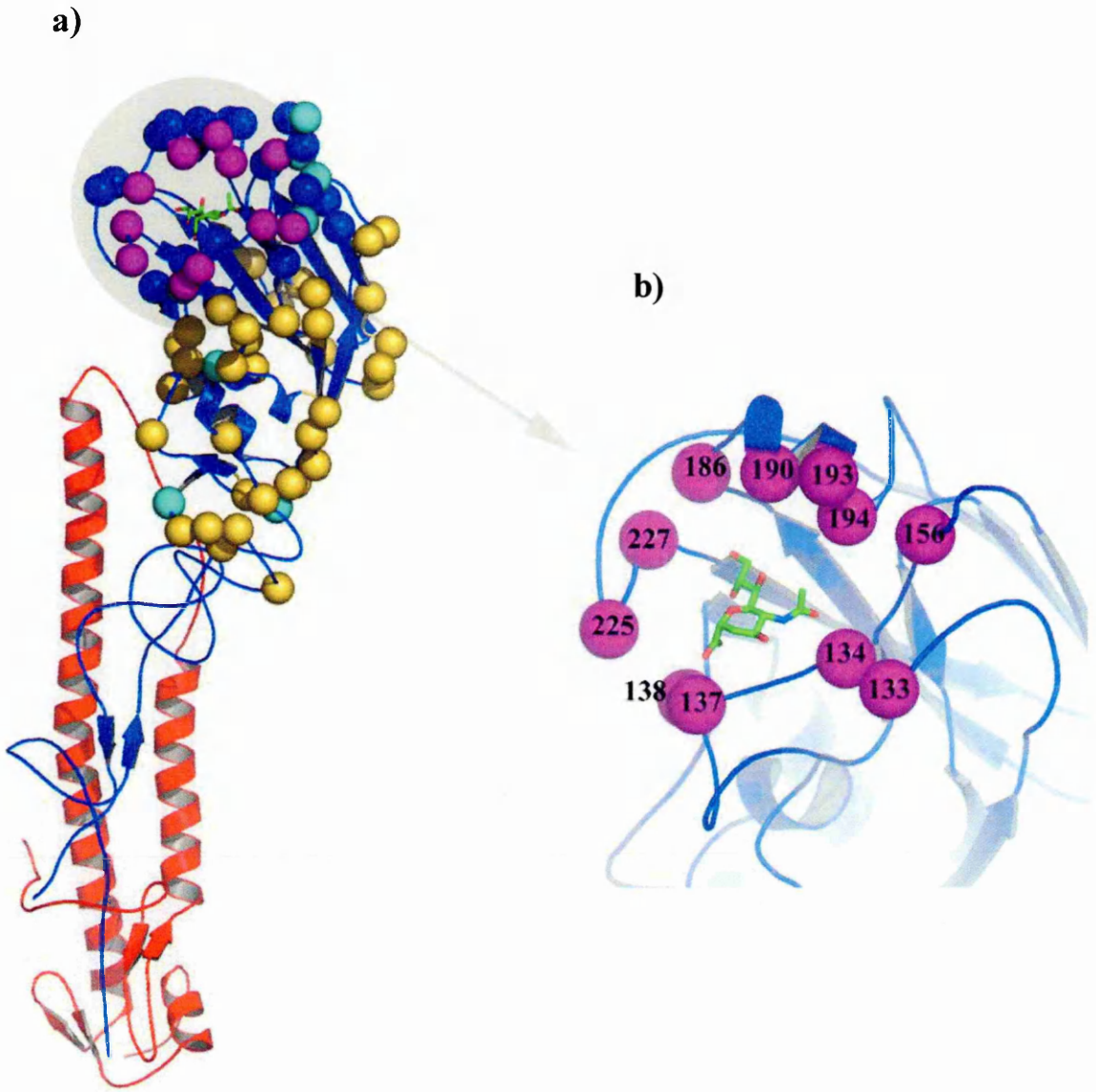


Figure 36 Schematic diagram showing residues containing changes in HA_1 for H1 viruses used in this study

a) HA monomer showing the location of all the changes. The different colours represent the classification of the residues. Circles in pink are residues located in the receptor-binding site (residues shaded in black in Table 10a). Circles in blue are residues located within 20 Å of the receptor-binding site or residues that are in a position to affect interaction with receptor analogues bound to neighbouring monomers. Circles in cyan are carbohydrate attachment sites. Circles in yellow are residues located elsewhere in HA_1.**b)** Close-up of the receptor-binding site and the residues at which changes are observed. Bound Neu5Ac is coloured in green.

host cell-mediated variation.

Whereas the Asp225Gly change has been shown to lead to an increased binding of α(2,3)-linked Neu5Ac, the Asp225Asn substitution does not appear to affect the affinity for 3'SL and 6'SLN (Gambaryan et al., 1999; Mochalova et al., 2003) (see Table 3). However, although associated with cultivation in eggs, Gly225 might also be representative of the original clinical isolate. Evidence for this finding is provided by the observed heterogeneity at this residue for the 1918 viruses isolated from people who died in the Spanish pandemic. Of these, 3 contained Asp and 2 Gly (Reid et al., 2003). Furthermore, evidence has been provided that the Asp225Gly substitution is involved in escape from mAb (Caton et al., 1982; Yates et al., 1990).

A single substitution Asp190Asn has been shown to lead to an increased affinity for the α(2,3)-linkage and a decreased affinity for the α(2,6)-linkage (Gambaryan et al., 1999). However, all the viruses used in this study of 1999-2003 contain Asn190 and are clearly α(2,6)-linkage specific (see Figure 35), which is in disagreement with the egg-adaptation studies. Furthermore, residue 190 is not likely to be associated with egg-adaptation for these isolates, since other viruses since 1999 were found to contain Asp190 or Asn190, irrespective of whether they were isolated from eggs or MDCK cells (WIC, London, in-house sequencing). Substitutions at position 190 have also been selected by mAb, which suggests the involvement of this residue in escape from immune surveillance (Nakajima et al., 1983).

The loss of a carbohydrate attachment site at 163, caused by the mutation Asn → Lys/Ser, is thought to lead to a loss of steric hindrance with more distal parts of receptors containing α(2,3)-linked sialic acid (Gambaryan et al., 1999). However, it appears that the α(2,3)-linkage recognition is not greatly affected by residue 163, since both Denver/1/57 and Finland/9/57 are clearly α(2,3)-specific and contain a glycosylation site at 163.

As can be seen in Table 3, the effects on receptor-binding properties of the remaining potential egg-adaptation mutations for the viruses used in this study (at residues 129, 138 and 189) have not been tested.

4.4 Discussion

4.4.1 H3 Subtype Viruses

4.4.1.1 Isolates Differ in their Receptor-Binding Properties and Antigenicity

A number of viruses from 1994-2003 were probed for their interaction with receptor analogues containing either α(2,3)- or α(2,6)-linked sialic acid by SPR. The data described here shows that evolution of H3 subtype viruses is not only associated with antigenic variation (see Table 4) but also with changes in affinity/specificity for host cell receptors (see Table 5 and Figure 32). Notably, the Fujian-like viruses, which emerged in 2002 and are antigenically different from previously circulating Panama/2007/99-like viruses, display unique receptor-binding properties by having a large decrease in affinity for α(2,3)-fetuin. As a consequence, these viruses display a large shift in specificity for the α(2,6)-linkage compared to the other isolates. In addition, they also show considerable variation (up to 200-fold) in binding strength for α(2,6)-fetuin. The remaining viruses are characterised by less marked differences in affinity for both α(2,6)- and α(2,3)-fetuin (maximum of ~ 6-fold for α(2,6)-fetuin). This indicates that antigenic variation can be but is not necessarily accompanied by large changes in receptor-binding properties.

Comparison of the HA_1 amino acid sequence determined in this study reveals that many residues, at which substitutions are observed, are located in close vicinity of the RBS. Since many of the antigenic sites have been shown to cluster around the RBS (see *1.7.5.2.1 Antigenic Sites, p.54*), the detected changes are most likely involved in escape from immune surveillance. The accumulation of changes close to the RBS indicates that

this area is favoured by positive selection in the human population, as suggested previously (Wiley et al., 1981; Wilson and Cox, 1990; Bush et al., 1999b). Since the viruses used differ in a number of residues, it is not possible to clearly identify the involvement of each individual substitution in the observed changes in antigenicity and/or receptor-binding properties. Nevertheless, the locations of a number of substitutions coincide with changes previously selected by mAbs for X31 (underlined residues in Table 6), indicative of their involvement in escape from antibody-recognition (Wiley et al., 1981; Underwood et al., 1987; Smith et al., 1991; Temoltzin-Palacios and Thomas, 1994; Patera et al., 1995).

Notably, a number of the observed substitutions are located directly in the RBS, in agreement with earlier reports (Cox and Bender, 1995; Nobusawa et al., 2000; Medeiros et al., 2001). Changes located in the RBS are likely to modulate receptor-binding properties either by directly affecting atomic contacts with cellular receptors or by causing small differences in the structure of the RBS. Why substitutions in the RBS occur is not clear. A number of studies indicate that adsorptive mutants arise when the antibody pressure is provided at sub-neutralising conditions (Fazekas de St. Groth, 1977; Yewdell et al., 1986; Temoltzin-Palacios and Thomas, 1994) or when the antibody is of low-affinity (Laeeq et al., 1997). Since binding to antibodies is in competition with attachment of virus to cells, modulation of HA affinity and/or specificity for cellular receptors might be sufficient to escape neutralisation by antibodies. Interestingly, infection by a novel antigenic variant has been reported to lead to the production of antibodies of reduced affinity, which is thought to be due to concomitant immunity to earlier strains (Fazekas de St. Groth and Webster, 1966). Therefore, such an alternative mechanism to antigenic variation for escape from neutralising antibodies might operate in the human host. Furthermore, it has been suggested that changes in the RBS might be due to structural constraints in antigenic sites

(Temoltzin-Palacios and Thomas, 1994). Alternatively, in addition to receptor-binding, residues in the RBS might also be directly involved in antigenic drift if their side-chains are surface-exposed. In order to clearly show the involvement of a particular residue in immune-evasion, selection of variants with a substitution at this residue would have to be selected with mAbs. Then, a decrease in affinity of this virus for the selecting mAb would have to be shown by ELISA, in order to directly demonstrate the role of this residue in antibody-recognition. If the antibody binds with similar affinity than to wild-type virus, the selected variant is more likely to be a receptor-binding mutant. Furthermore, since the viruses differ from each other by a number of residues, the possibility that certain substitutions occur in order to compensate for a structural change close to or in the RBS can not be excluded.

It has been generally believed that RBS residues need to be conserved in the process of antigenic drift in order to maintain receptor-binding function (Both et al., 1983b; Weis et al., 1988; Bizebard et al., 1995). However, the fact that substitutions in the RBS do occur indicates that the plasticity of the RBS is greater than expected.

4.4.1.2 Decreased Affinity for α(2,3)-Fetuin of Fujian-like Viruses

All the viruses used in this study isolated since 2002 displayed a marked decrease in affinity for the α(2,3)-fetuin compared to the earlier viruses. The reduced binding strength for this receptor analogue correlated with poor growth of these viruses in eggs, which presented problems in their isolation. Since allantoic cells have been shown to contain more α(2,3)- than α(2,6)-linked sialic acid (Ito et al., 1997b), the low virus yield from eggs could be a result of the low affinity for the α(2,3)-linkage.

Sequence comparison revealed that the Fujian-like viruses are distinguished from the other isolates by the same substitution as the single-site X31 mutant Gly225Asp. This

virus, which was initially used to test the sensitivity of the SPR assay (see *Chapter 3*), exhibited a similar reduction in binding strength for α(2,3)-fetuin compared to X31 as the recent viruses. This finding strongly implies that Asp225 is responsible for the reduced affinity for α(2,3)-fetuin. The structural basis for this interaction is not obvious. Although this residue is located directly in the RBS, no direct contacts of this amino acid with the glycosidic linkage or more distant moieties of receptor analogues have been revealed in structural studies for H3 HA (see Figures 7 and 8, p.42-43). This suggests that the negative effect of Asp225 on the interaction with α(2,3)-fetuin is of indirect character. Since these viruses all contain a glycosylation site at residue 165, an oligosaccharide attached at this position would be projected towards asialo-components of receptor analogues containing α(2,3)-linked but not α(2,6)-linked sialic acid (see Figure 37). It might be speculated that the Gly225Asp causes a rearrangement of the oligosaccharide so as to interfere with binding to receptors containing α(2,3)-linked sialic acid. Furthermore, the Fujian-like viruses all contain a concomitant change Trp222Arg compared to other isolates. The close location of residues 222 and 225 would enable a salt-bridge between the two. The observation that the Fujian-like viruses bind α(2,3)-fetuin more weakly compared to the Gly225Asp mutant might be a result of further structural rearrangements caused by the two substitutions. The ability of oligosaccharides at the membrane-distal part of HA to affect receptor-binding properties has been shown in a number of studies (Deom et al., 1986; Matrosovich et al., 1997; Ohuchi et al., 1997; Abe et al., 2004). The effect of a specific carbohydrate on linkage-recognition has been reported previously for USSR/90/77 and Tübingen/12/85 (H1N1) (Gunther et al., 1993; Gambaryan et al., 1998). These studies have shown that variant viruses containing an oligosaccharide attachment site at residue 131 were distinguished by those without this site by a reduced affinity for receptor analogues containing α(2,6)-linked sialic acid. However, the contribution of other residues

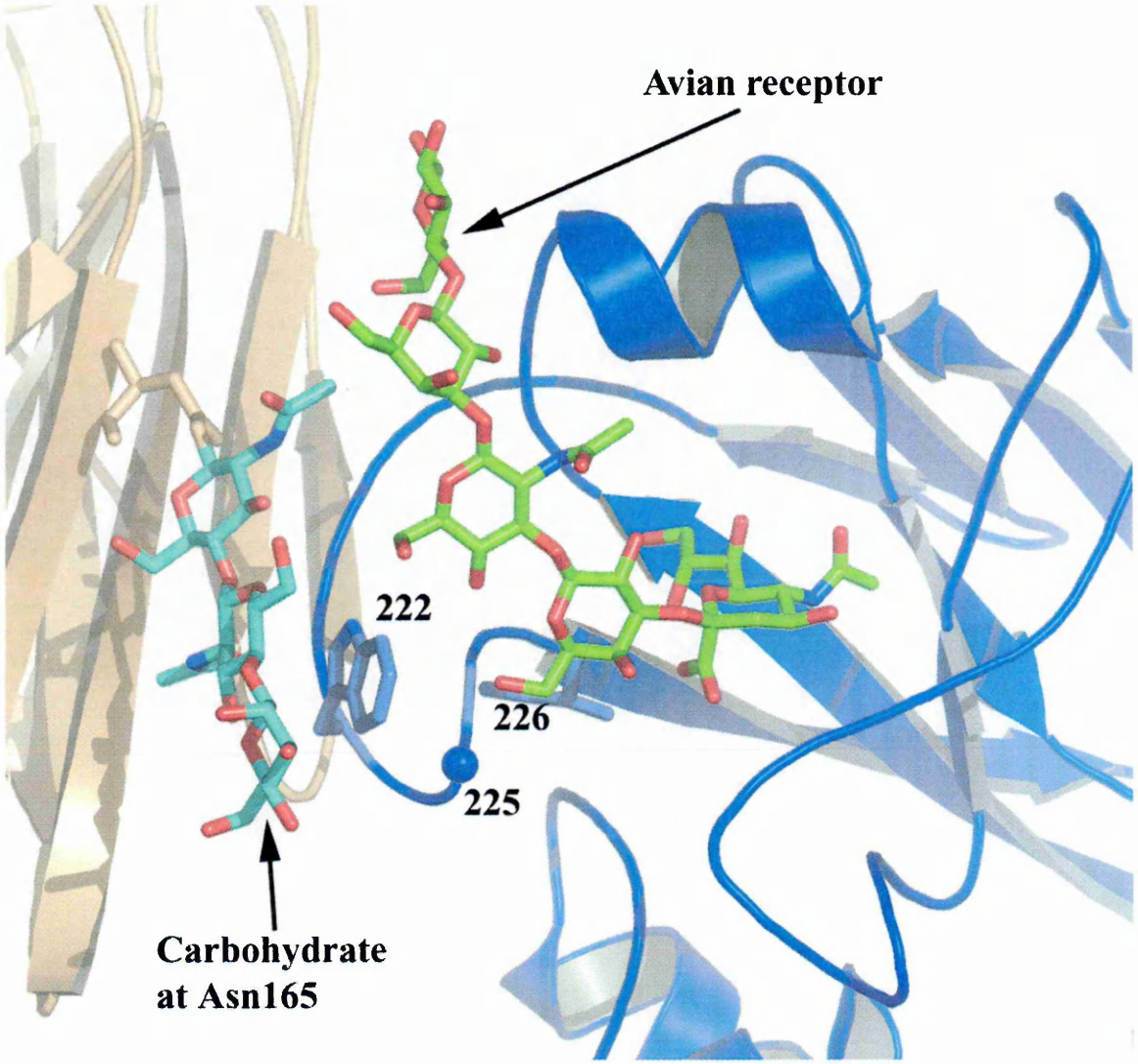


Figure 37 Model for reduced affinity of Fujian-like H3 HA for α(2,3)-fetuin
Schematic diagram showing the close proximity of a carbohydrate at Asn165 protruding from the monomer on the left (coloured in grey) to a α(2,3)-linked receptor analogue bound to the neighbouring monomer (coloured in blue). Residues 222 and 225 could potentially influence the position of the carbohydrate, thereby affecting its interference with distant parts of α(2,3)-linked receptor analogues ("avian" receptor, coloured in green).

to the detected changes in receptor-binding properties for the Fujian-like viruses should not be excluded.

The implications of the reduced affinity for the α(2,3)-linkage with regard to infection of human host cells is not clear. It is generally believed that the ability to infect human cells of the respiratory tract correlates with the recognition of α(2,6)-linked sialic acid, and that the observed α(2,6)-linkage specificity of human influenza viruses is a result of the selection pressure exerted by these cells (see *1.7.4.4 Host Cell Factors Involved in Selection of Linkage Specificity, p.49*). However, mucins, produced by human epithelial cells, are rich in α(2,3)-linked sialic acid (Breg et al., 1987; Baum and Paulson, 1990). Since mucoproteins and mucopolysaccharides have been shown to inhibit the virus' ability to agglutinate RBC (Burnet, 1951), reduced binding to these has been suggested to play a role in the evolution of receptor specificity towards recognition of the α(2,6)-linkage. The reduced affinity for α(2,3)-linked Neu5Ac observed for the recent Fujian-like isolates might therefore facilitate the infection of human host cells. This raises the possibility that the substitutions leading to the reduced binding strength for α(2,3)-linked Neu5Ac were selected by host cell-specific factors other than antibodies and reflect further adaptation to infection of the human host. Alternatively, the amino acid differences of the Fujian-like viruses compared to the earlier strains might be the result of antigenic variation leading to a concomitant effect on receptor-binding properties.

The finding that the H3 virus isolates of 1968-1999 used in this study interacted with α(2,3)-fetuin indicates that the acquirement of α(2,6)-linkage recognition for the initial transfer from birds to humans was not coupled with a loss of binding to the α(2,3)-linkage. Thus, the SPR data indicate that, although the possible 1968 pandemic precursor (Dk/Ukraine/1/63) already recognised the human-specific α(2,6)-linkage, the first H3 virus isolated during the pandemic (represented by X31) displayed an almost 100-fold higher

affinity for α(2,6)-fetuin. In contrast, only about a 5-fold decrease in affinity for the avian-specific α(2,3)-linkage was observed for X31 compared to Dk/Ukraine/1/63.

4.4.1.3 Distinct Receptor-Binding Properties of Christchurch/28/03

The weak binding strength for α(2,3)-fetuin renders the Fujian-like viruses highly α(2,6)-specific, reflected in their specificity indices being > 200 (see Table 5). This could give the impression that these viruses display high affinity for α(2,6)-fetuin. However, the Fujian-like viruses were shown to vary greatly with regard to α(2,6)-fetuin affinity. Notably, these viruses bound α(2,6)-fetuin generally more weakly compared to the earlier isolates, e.g X31. The only exception to this is Christchurch/28/03, which bound this receptor analogue as strongly as X31. Christchurch/28/03-specific changes in close vicinity to the RBS with the potential to affect receptor-binding properties are at residues 126, 138, 186 and 190 (see Table 6a). The loss of a glycosylation site at 126 is not likely to account for the increase in affinity for α(2,6)-fetuin compared to the other Fujian-like viruses, since Wyoming/3/03 is also not glycosylated at this site due to a change at position 128.

Residue Glu190 in H3 HA has been shown to directly contact the 9-OH moiety of Neu5Ac via a hydrogen bond (see Figure 6, p.37) (Weis et al., 1988). All the viruses used in this study contain Asp190, except for Christchurch/28/03, which contains Val190. It is possible that Asp, despite its smaller size compared to Glu, might still form a hydrogen bond to 9-OH via a water molecule. In contrast, Val190 would not be able to make any contacts to Neu5Ac, which would be expected to lead to a decrease in affinity. However, a change from Glu190 to Ala, which would also not be able to contact the 9-OH, has been reported to lead to a slight increase of affinity for human RBC (Martin et al., 1998). In addition, a Glu190Asp change has been implicated in the inability of viruses isolated since 1992 to agglutinate chicken RBC (Nobusawa et al., 2000). Interestingly,

Christchurch/28/03 contains Asp186, in contrast to the other Fujian-like viruses containing Val or Gly at this position. Asp186 might be close enough to 9-OH to form a hydrogen bond and could therefore potentially take over the role of Glu190. This might explain the general increase in affinity of Christchurch/28/03 for both α(2,3)- and α(2,6)-fetuins compared to the other Fujian-like viruses. Finally, residue 138 points towards residue 226 and could potentially affect its position, thereby affecting receptor-binding properties.

Interestingly, Christchurch/28/03 does not only differ from the other Fujian-like viruses by substitutions in HA but also in NA, which can be distinguished phylogenetically (WHO, 2004). Christchurch/28/03 contains a NA closely related to that of New York/55/01 (Panama/2007/99-like virus). It is therefore likely that Christchurch/28/03 obtained its NA by reassortment. Since a functional balance between HA and NA has been suggested to be important for optimal virus replication (see *1.10 Functional Balance between HA and NA Activity, p.66*), investigation of a possible correlation of receptor-binding and enzyme activity properties would be of interest.

4.4.1.4 Role of Residue 226 in Defining Linkage Specificity

As described in *1.7.4.2 Correlation between Receptor-Linkage Specificity and Host of Origin, p.39*, residue 226 was shown to be a major determinant for defining receptor specificity for the H3 subtype. Human isolates contain Leu/Val/Ile and are α(2,6)-specific, whereas avian isolates contain Gln226 and are α(2,3)-specific (Connor et al., 1994; Lindstrom et al., 1996; Mori et al., 1999). However, Johannesburg/33/94 contained the avian-typical Gln226. Although the Gln226 is likely to be the result of egg-adaptation and therefore not representative of the original clinical isolate, the decreased α(2,6)-fetuin affinity of Johannesburg/33/94 compared to X31, Sydney/5/95 and Panama/2007/99 shows the involvement of residue 226 in modulating linkage recognition. However, Gln226 did

not cause a switch in receptor specificity, as observed for the Leu226Gln substitution for X31 (Rogers et al., 1983a). Therefore, additional residues appear to be important in defining receptor specificity. For example, as described above, residues 222 and 225 rather than 226 are suggested to be implicated in the large shift in specificity for the Fujian-like viruses.

4.4.1.5 Comparison of SPR Data with Previously Reported Results

Little information on receptor-binding properties of the viruses used in this study is available in the literature, except for Dk/Ukraine/1/63 and X31. The affinity data of X31 was already discussed in *Chapter 3* and is in general agreement with results from other studies. The α(2,3)-linkage specificity of Dk/Ukraine/1/63 described here is also consistent with the linkage preference of this virus determined by various assays (see Appendix 3, p.257). However, as for X31, considerable variation can be observed when the relative affinity differences between the α(2,3)- and the α(2,6)-linkages are compared, ranging from less than 2-fold (Nobusawa et al., 1991) to 60-fold (Matrosovich et al., 1993; Matrosovich et al., 2000). The SPR results of this study are in agreement with the latter value (see Table 5). As discussed in *Chapter 3*, the variation in relative affinity is probably due to differences in the assays, such as the choice of receptor analogues and their valency. The only other virus for which receptor-binding data was available is Sydney/5/97. A haemagglutination assay with RBC reconstituted with Neu5Ac either in the α(2,3)- or α(2,6)-linkage suggests α(2,6)-linkage specificity for this virus, with HAUs differing 2-fold (Medeiros et al., 2001). The relative affinity difference for Sydney/5/97 was ~ 4.5-fold as determined by SPR, which is in good agreement with the haemagglutination assay.

4.4.2 H1 Subtype Viruses

4.4.2.1 Antigenically Distinct H1 Isolates Display Differences in Receptor-Binding Properties

Receptor-binding properties in relation to antigenic drift were also studied for H1 subtype viruses isolated between 1934 and 2003. This virus was introduced in 1918 and caused the biggest human pandemic reported in the 20th century (see *1.8.1 Origin of Pandemic Influenza Viruses, p.61*). After introduction of the H2 subtype in 1957 they were absent from the human population until 1977, when they suddenly reappeared. Since then they continue to circulate together with the H3 subtype, which replaced the H2 subtype in 1968. It was therefore of interest to investigate whether viruses from the first epidemic period differed from the isolates of the second era with regard to affinity and/or specificity for receptor analogues.

The SPR data reveal great variations in affinities for α(2,3)- and α(2,6)-fetuin for viruses of 1934-2003. Importantly, in contrast to the general α(2,6)-linkage preference of the human H3 viruses studied, a number of H1 isolates display either clear α(2,3)-linkage specificity or do not significantly distinguish between α(2,3)- and α(2,6)-fetuin ("dual"-linkage specificity) (see Figure 35). Notably, the majority of the viruses from the first epidemic period were shown to be α(2,3)-specific, namely the earliest isolate Puerto/Rico/8/34 and the viruses of 1952-1957. The remaining viruses of this period, including the swine isolate, appear to be "dual"-linkage specific. The viruses of the second epidemic era of 1978-1999 era are also either α(2,3)- or "dual"-linkage specific. Interestingly, all the viruses since 1999 are clearly "human"-like, as they are characterised by a clear α(2,6)-linkage preference (see Figure 35). In addition, these viruses are all antigenically closely related (see Table 8), which is in agreement with the reported slow antigenic evolution of 1999-2003 (Hay et al., 2001). Therefore no significant changes in

either antigenic or receptor-binding properties have occurred since 1999. Slow antigenic evolution has also been reported between 1986-1995 (Hay et al., 2001), which is supported by the antigenic similarity for Taiwan/1/86 and Bayern/7/95 (see Table 8). Since these viruses also display similarities in their interaction with α(2,3)- and α(2,6)-fetuin, (see Figure 35), an interrelationship between antigenic and receptor-binding properties is suggested. However, as noted earlier for the H3 viruses, the extent to which changes in antigenicity correlate with changes in the interaction with receptor analogues appears to vary.

Very similar receptor-binding properties were observed for Denver/1/57 and Finland/9/57, which differed from each other by only one residue, and for Bucharest/955/03 and Prague/9/03, which displayed identical HA_1 amino acid sequences. This finding strongly indicates the robustness of the SPR assay.

4.4.2.2 Residues Implicated in the Observed Variation of Receptor-Binding Properties

As for the H3 viruses, the H1 isolates differ at many residues close to the RBS (see Table 10 and Figure 36). This finding makes it difficult to assess the involvement of each residue in defining antigenic and receptor-binding properties. Almost all of these could potentially be involved in antigenic variation, because their side-chains are surface-exposed. Substitutions at a number of these residues have been selected by mAb, which suggests their direct involvement in escape from neutralising antibodies (Caton et al., 1982; Nakajima et al., 1983; Yates et al., 1990). Notably, differences are observed at residues located directly in the RBS. These appear to correlate with the observed variation in both receptor-binding and antigenic properties for viruses of 1934-1999. A number of RBS residues, at which differences are observed, have previously been suggested to play a role

in defining receptor specificity, namely residues 138, 186, 190, 194 and 225 (Rogers and D'Souza, 1989; Matrosovich et al., 1997; Gambaryan et al., 1999; Mochalova et al., 2003). Residues 225 and 190 have also been reported to be associated with propagation of viruses in eggs (see Table 3). Studies of egg-adaptation mutations of H1 viruses have also provided evidence for the involvement of residues 190 and 225 in modulation of receptor specificity (Gambaryan et al., 1999; Mochalova et al., 2003). In contrast, the viruses since 1999 not only display similar receptor-binding and antigenic properties, but also identical RBS residues, apart from a few amino acids for Egypt/96/02 (see below).

The viruses used in this study contain Glu, Asp or Asn at position 190, with the exception of Beijing/262/95, which contains Val190. Glu190 is highly conserved for all avian subtypes, whereas human isolates have been reported to generally contain Asp190 (Matrosovich et al., 1997). Interestingly, all the human isolates containing the avian-typical Glu190 displayed clear α(2,3)-linkage preference, whereas all the viruses containing Asn190 were clearly α(2,6)-specific (see Figure 35). This finding suggests a role of this residue in defining linkage preference. The structure of Sw/Iowa/15/30 HA complexed with a receptor analogue containing Neu5Ac in the α(2,6)-linkage reveals a hydrogen bond between Asp190 and GlcNAc-3 (see Figure 12, p.48). This additional contact might stabilise the accommodation of α(2,6)-linked Neu5Ac, leading to an increase in affinity. On the other hand, Asp190 does not appear to prevent binding of Neu5Ac in the α(2,3)-linkage, based on the observation that the viruses used in this study with Asp190 were either "dual"- or α(2,3)-linkage specific. Therefore, Asp190 does not necessarily confer α(2,6)-linkage specificity. It could be speculated that the α(2,3)-linkage specificity of isolates containing Glu190 would be the result of the inability of this amino acid to provide the additional contact to receptors containing α(2,6)-linked sialic acid observed for Asp. How Val190, as in the case for Beijing/262/95, would affect receptor-binding

properties, is not clear. However, a more passive role is predicted, since Val is not able to provide contacts to any parts of sialic acid-containing receptors. The structural basis for the contribution of Asn190 for the α(2,6)-linkage preference of the viruses of 1999-2003 is yet unknown. However, Asn is similar in size and polarity to Asp and might therefore also be able to form a stabilising hydrogen bond to GlcNAc-3 of receptors containing α(2,6)-linked sialic acid. The correlation of Asn190 and α(2,6)-linkage specificity for the viruses used in this study is in disagreement with an earlier study, where an Asp190Asn substitution led to an increase in affinity for the α(2,3)-linkage with a concomitant decrease in affinity for the α(2,6)-linkage, resulting in "dual"-linkage specificity (Gambaryan et al., 1999) (see Table 3).

Residue 225 was either Asp or Gly, with the exception of Chile/1/83 containing Asn at this position. The involvement of residue 225 in linkage specificity is based on studies showing that an Asp225Gly substitution can lead to an increase in affinity for the α(2,3)-linkage without markedly affecting affinity for the α(2,6)-linkage for monovalent receptors (Gambaryan et al., 1999). When a multivalent receptor analogue was used, the increase in affinity for the α(2,3)-linkage was greater than 500-fold, leading to very similar affinities for both the α(2,3)- and α(2,6)-linkage (Mochalova et al., 2003). In contrast, an Asp225Asn did not greatly affect the recognition of either linkage. No strict correlation could be observed for amino acid 225 and receptor-binding properties for the H1 viruses in this study. Whereas the "dual"- and α(2,3)-linkage specific viruses contained either Gly or Asp at 225 (with the exception of Asn for Chile/1/83), all the α(2,6)-specific viruses contained Asp225. X-ray studies of Puerto Rico/8/34 complexed with receptor analogues have shown that Asp225 can be directly involved in the interaction with the α(2,6)-linkage by forming a hydrogen bond to Gal-2. In contrast, due to the lack of a side-chain, Gly225 of Sw/Iowa/15/30 is unable to contact any parts of this receptor analogue (see Figures 11

and 12, p.47-48).

Residue 138 has been suggested to play a role in linkage-recognition, based on the observation that many of the early H1 isolates containing Ala138 were "dual"-linkage specific, whereas later isolates containing Ser138 were α(2,6)-linkage specific (Rogers and D'Souza, 1989). Indeed, all the viruses used in this study with Ala138 were either "dual"- or α(2,3)-specific, whereas all the 1999-2003 viruses contain Ser138 and are α(2,6)-specific. However, Ser138 is not strictly correlated with α(2,6)-linkage specificity, since Brazil/11/78 with Ser138 is "dual"-linkage specific. This suggests that linkage specificity is defined by a combination of amino acids at different residues. No structural evidence has as yet been provided for the contribution of residue 138 to modulation of receptor-binding properties.

Residue 194 has been suggested to be involved in receptor-recognition due to the fact that it is a highly conserved Leu in avian subtypes, whereas human H1 isolates usually contain Ile at that position (Matrosovich et al., 1997). However, the majority of the viruses used in this study contain the avian-typical Leu194. Furthermore, since the viruses containing Leu194 were either α(2,3)-, "dual"- or α(2,6)-linkage specific, residue 194 is not likely to play an important role in defining linkage specificity. Similarly, residue 186 has been implicated in modulation of receptor-binding properties because it was shown to be a highly conserved Pro in avian H1 viruses, whereas most human isolates contained Ser186 (Matrosovich et al., 1997). However, all the viruses used in this study of 1999-2003 contain the avian-typical Pro186 and are α(2,6)-linkage specific, whereas Puerto Rico/8/34, which also contains Pro186, is α(2,3)-specific. Residue 186 is therefore unlikely to be a major determinant for linkage specificity.

In summary, it appears that receptor specificity is defined not by single residues but by a combination of different amino acids. The only exception to this was Glu190, which

strictly correlated with α(2,3)-linkage specificity. In order to address the contribution of residues 138, 190, 194 and 225 to linkage-recognition on a structural basis, X-ray studies of HA in complex with receptor analogues would have to be performed for a number of H1 viruses used in this study.

As described in *4.3.4 Assessment of Egg-Adaptation Mutations, p.166*, it is not clear whether the α(2,3)- and "dual"-linkage specificity of isolates from 1934-1999 are representative of the original clinical specimen or an artefact of extensive laboratory passage of these viruses in eggs. Notably, the 1918 pandemic virus was reported to be α(2,6)-specific (Kobasa et al., 2004), which is in contrast to the α(2,3)-linkage specificity of Puerto/Rico/8/34. As mentioned above, the α(2,3)-linkage preference of Puerto/Rico/8/34 might be due to an avian-typical Glu190, whereas the 1918 isolates were reported to contain the human-typical Asp190 (Reid et al., 2003).

4.4.2.3 Distinct Receptor-Binding Properties of the H1N2 Reassortant Virus

Antigenic evolution has been reported to be generally slower and less marked for the H1 subtype than the H3 viruses since 1977, despite their similar rate of genetic evolution. This observation is reflected in the fewer changes in the vaccine composition for H1 viruses (Hay et al., 2001). In addition, the emergence of antigenic variants has been observed to correlate with the predominance in epidemics (Lin et al., 2004). The less frequent and slower emergence of antigenic variants for the H1 viruses is therefore also reflected in the infrequent predominance over H3 and B viruses, with prevalence only observed in 1988-1989 and 2000-2001. Following the 2000-2001 epidemic season, the H1N2 reassortant viruses appeared. These contain an HA antigenically and genetically similar to New Caledonia/20/99 and a NA and six internal genes closely related to contemporary H3N2 viruses (Gregory et al., 2002). It was suggested that HA amino acid changes accumulated

between 1995-1999 might have facilitated the replacement of H3 by H1 HA, in order to functionally match the N2 NA activity. The acquired α(2,6)-linkage preference of all the viruses since 1999, including the H1N2 reassortant Egypt/96/02, show that the accumulated changes indeed had a large effect on receptor-binding properties (see Figure 35). Furthermore, it was observed that Egypt/96/02 differs from the other 1999-2003 viruses by a 10-fold higher affinity for α(2,6)-fetuin and a 100-fold lower affinity for α(2,3)-fetuin. As a result, this virus displays a higher α(2,6)-linkage preference compared to the other viruses.

Amino acid sequence comparison revealed that Egypt/96/02 differs from the other 1999-2003 isolates at residues 132, 193 and 218. Since Egypt/96/02 is antigenically closely related to the other recent isolates, these changes are most likely to account for the observed differences in receptor-binding properties. Ser193 in H1 viruses has been shown to contact Gal-4 of a receptor analogue with α(2,6)-linked Neu5Ac by forming a hydrogen-bond with the hydroxyl of its side-chain (Gamblin et al., 2004) (see Figure 12, p.48), which might result in an enhanced interaction with this receptor analogue. Egypt/96/02 contains Thr at 193 which, unlike the other viruses containing Ala at this position, would be able to contact receptor analogues with α(2,6)-linked Neu5Ac in the same manner. In addition, changes at residue 193 were reported to modulate binding to derivatised RBC containing α(2,3)-linked Neu5Ac for H3 viruses (Daniels et al., 1987). The increased binding to α(2,6)-fetuin and the decreased α(2,3)-fetuin affinity for Egypt/96/02 might therefore be a result of Thr193. Residue 218, which is in the trimer interface, has also been shown to modulate receptor-binding properties, namely the interaction with α(2,3)-linked Neu5Ac (Daniels et al., 1987). The structural basis for this finding is unknown. In contrast, due to its location at the edge of the RBS, residue 132 is less likely to influence interactions with receptors.

The low affinity of the H1N2 reassortant virus for α(2,3)-fetuin was observed to be very similar to that of the recent H3 viruses (see Figures 32 and 35). These distinct receptor-binding properties correlate with the presence of a closely related N2. Since it has been suggested that HA receptor-binding and NA receptor-destroying functions need to be balanced for optimal virus replication (see *1.10 Functional Balance between HA and NA Activity, p.66*), the changes in receptor-binding properties of the H3 and H1N2 virus might interrelate with changes in NA activity and/or specificity. The substitutions in Egypt/96/02 compared to the other H1N1 HAs might therefore have been necessary to functionally match N2 NA activity. A possible interrelationship between the observed receptor-binding properties of recent H3 viruses and H1N2 viruses would be addressed by studying the NA activity and specificity of theses viruses.

4.4.2.4 Comparison of SPR Data with Previously Reported Results

Receptor-binding data on the viruses used in this study was available for Puerto Rico/8/34, Dk/Alberta/35/76, Sw/Iowa/15/30, Fort Monmouth/1/47, Fort Warren/1/50, Fort Leonard Wood/1/52, Denver/1/57, Brazil/11/78, Chile/1/83 and Taiwan/1/86 (see Appendix 3, p.257). A number of the results reported in the literature were conflicting, which makes comparison with the SPR data difficult. For example, Puerto Rico/8/34 was determined to be either α(2,3)-specific, "dual"-specific or α(2,6)-specific, depending on the study. The SPR data reported here are in support of this virus displaying α(2,3)-linkage preference. The differences in results might be due to the presence of different variants of this virus, which are thought to have arisen by independent laboratory passages from the original source. For example, whereas some viruses contain an oligosaccharide at position 131 (Cambridge strain), the strain used in this study (Mount Sinai strain) is characterised by a loss of the glycosylation site due to a deletion at residue 132 (Winter et al., 1981; Caton et

al., 1982). Furthermore, variants of this virus containing either Asp190 or Glu190 are available, which are likely to affect receptor specificity (see Influenza database, http://www.flu.lanl.gov/). The lack of information on the identity of the Puerto Rico/8/34 strain used in the reported assays does not allow the comparison of results. Furthermore, as discussed in *Chapter 3*, variations in relative affinities might also be due to differences in the assays, such as the choice of receptor analogues and the valency of these. Despite these difficulties in data comparison, the SPR data are in general agreement with previous results. In particular, the "dual"-specificity of the H1 viruses of the first epidemic era has been reported before, as was the α(2,3)-linkage specificity of Denver/1/57 and Fort Leonard Wood/1/52 (Rogers and D'Souza, 1989; Matrosovich et al., 1993).

5 Substitutions in Avian H3 HA Associated with Selection by Derivatised RBC containing α(2,6)-linked Neu5Ac

5.1 Introduction

The Hong Kong pandemic (H3N2) in 1968 was caused by a reassortant virus introducing a novel HA into the human population (see *1.8 Emergence and Evolution of Human Influenza Viruses, p.61*). Initially, either an equine or avian origin of HA was proposed based on antigenic analyses of isolates in comparison with strains recovered from horses and birds (Coleman et al., 1968; Tumova and Easterday, 1969; Zakstelskaja et al., 1969; Laver and Webster, 1973). Subsequent sequence and phylogenetic studies were in support of an avian origin of HA (Fang et al., 1981; Ward and Dopheide, 1981; Bean et al., 1992). Although the identity of the bird virus involved in the reassortment has not been identified, the progenitor virus was closely related to Dk/Ukraine/1/63. The earliest human H3 pandemic isolate (Aichi/2/68) differs from Dk/Ukraine/1/63 by only 15 amino acids in HA_1, of which 5 are located in the RBS (at positions 137, 193, 226, 227, 228) (Ha et al., 2003). Despite the small number of changes the two viruses display receptor-linkage preference typical of their host of origin (avian HA = α(2,3), human HA = α(2,6)) (Rogers and Paulson, 1983; Matrosovich et al., 1993; Matrosovich et al., 2000). The substitution Gln226Leu has been implicated in facilitating species transfer of the H3 subtype from birds to humans by shifting receptor-linkage preference from α(2,3) to α(2,6) (Matrosovich et al., 2000). The evolution of the contrasting linkage specificity of avian and human viruses is thought to have occurred by selection pressure exerted by the respective linkage of receptors on human and avian target cells (see *1.7.4.4 Host Cell Factors Involved in Selection of Linkage Specificity, p.49*). The main evidence for the involvement of residue

226 in linkage discrimination was provided by selection of X31 (reassortant virus containing the HA of Aichi/2/68) variants by means of propagation in the presence of a source of α(2,6)-linked sialic acid (horse serum) (Rogers et al., 1983a). These variants displayed a shift in linkage specificity from α(2,6) to α(2,3), which was attributed to a single mutation Leu226Gln. A further indication for the involvement of residue 226 in receptor specificity is that Gln226 is highly conserved across all avian HA subtypes, whereas human H3 viruses never contain Gln but always a non-polar amino acid (Leu, Val or Ile) (Nobusawa et al., 1991; Matrosovich et al., 1997; Medeiros et al., 2001).

Selection in the reverse direction has also been performed by means of adsorption to and elution from RBC with α(2,6)-linked Neu5Ac, generating a Dk/Ukraine/1/63 variant displaying α(2,6)-linkage preference typical of human isolates (Rogers et al., 1985). Although this variant was characterised by a substitution at residue 226 from Gln to Leu, this mutation reverted upon growth in eggs. In contrast, Aichi/2/68 HA and its single-site mutant Leu226Gln maintained residue 226 upon propagation in eggs. Structural comparison of HA reveals that, although the Dk/Ukraine/1/63 and Leu226Gln RBS are similar, small conformational differences are apparent (Ha et al., 2003) (see Figure 38). Since these viruses differ only at residues 137, 193, 227 and 228 in or close to the RBS, these are most likely to account for the observed conformational changes. It is possible that the small structural differences in the RBS would contribute to the inability of Dk/Ukraine/1/63 to maintain the Gln226Leu mutation upon growth in eggs.

Residues 137 193 227 and 228 all have the ability to affect receptor-binding properties. Human H3 viruses contain Ser228 in contrast to a conserved Gly228 for all avian subtypes (Nobusawa et al., 1991; Matrosovich et al., 1997). In the HA-receptor analogue complex, residue 228 interacts with the 9-OH moiety of Neu5Ac either directly (Ser228) or indirectly via a water molecule to its main chain (Gly228) in human H3 and

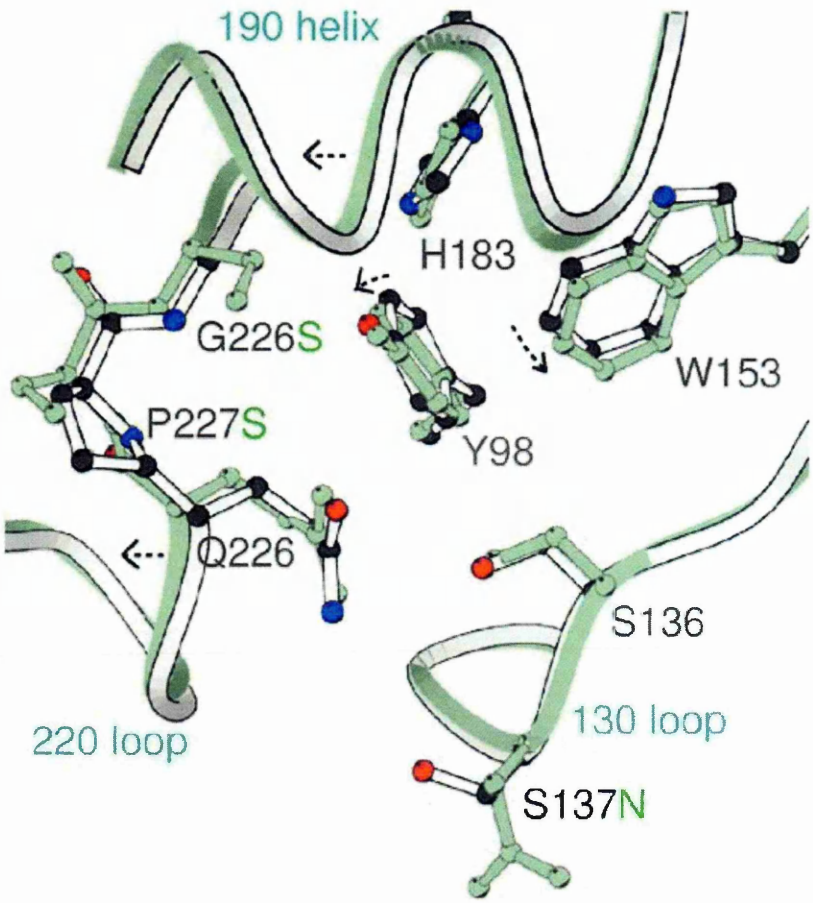


Figure 38 Superimposition of the receptor-binding sites of Dk/Ukraine/1/63 and Leu226Gln to show conformational differences Dk/Ukraine/1/63 HA residues are coloured in white and Leu226Gln in green. Side-chain and main-chain shifts are indicated by dashed arrows.

avian H5 HA, respectively (Weis et al., 1988; Ha et al., 2001). Although no contacts of this residue to any asialo parts of the receptor analogues have been observed, amino acid 228 has been implicated in affecting receptor specificity. A Ser228Gly mutation in the presence of Leu226 has been shown to lead to a slight increase in affinity for human RBC (Martin et al., 1998). Furthermore, the substitution Gly228Ser in the presence of Gln226 has been shown to lead to a decreased affinity for the α(2,3)-linkage (Matrosovich et al., 2000). This observation suggests that, while human H3 HA acquired the ability to recognise receptors containing α(2,6)-linked Neu5Ac by the Gln226Leu change, a concomitant Gly228Ser change would contribute to the α(2,6)-linkage specificity by reducing the affinity for the α(2,3)-linkage. This finding is in agreement with a human H3 virus containing a Leu226Gln mutation, which displayed an enhanced affinity for horse RBC by an additional Ser228Gly mutation (Vines et al., 1998). Horse RBC have been shown to contain mainly sialic acid in the α(2,3)-linkage and are not agglutinated by human but by avian viruses (Ito et al., 1997a). Furthermore, the Ser228Gly mutation, in addition to the Leu226Gln mutation in a human H3 HA, has been shown to be important for virus replication in duck intestine (Vines et al., 1998).

The presence of Pro at 227 for Dk/Ukraine/1/63 is unusual, since human and avian H3 HAs usually contain Ser at this position (Bean et al., 1992). Receptor-binding studies of avian H3 viruses have suggested that the Ser227Pro change causes a decreased affinity for the α(2,3)-linkage (Matrosovich et al., 2000). Residue 193 has also been shown to affect receptor-binding properties, since substitutions at this residue affected binding to Neu5Ac in both the α(2,3)- and α(2,6)-linkage (Daniels et al., 1987). Modulation of receptor-binding properties has not been demonstrated for residue 137, but since this residue is located directly in the RBS, it has the potential to affect interactions with sialic acid. In summary, the studies described above all stress the importance of residues other

than 226 in the modulation of receptor-binding properties.

The selection assay with Dk/Ukraine/1/63 performed by Rogers et al. (1985) was repeated with the aim to determine whether continued selection pressure to recognise α(2,6)-linked Neu5Ac would lead to additional changes in HA upon substitution of residue 226 from Gln to Leu. Selected variants would then be tested by SPR for changes in their receptor-binding properties. As well as Dk/Ukraine/1/63, viruses isolated from ducks in Hokkaido in the 1980s have been shown to be closely related to Aichi/2/68 (Kida et al., 1987; Bean et al., 1992). This finding raises the possibility that the HA progenitor for the pandemic strain was Dk/Hokkaido-like. In contrast to Dk/Ukraine/1/63 with Pro227, these viruses contain Ser227 typical for avian and human H3 isolates. Dk/Hokkaido/33/80 was therefore included in the selection assay, in order to elucidate the importance of residue 227 for genetic stability of acquired α(2,6)-linkage preference.

5.2 Receptor-Binding Properties of Dk/Hokkaido/33/80, Dk/Ukraine/1/63 and Aichi/2/68

Prior to selection of receptor-binding variants, the HA_1 amino acid sequence and receptor-binding properties of Dk/Ukraine/1/63 and Dk/Hokkaido/33/80 were compared. Sequence analysis revealed 13 differences between the two viruses (at residues 25, 63, 81, 102, 137, 145, 158, 160, 201, 227, 236, 303, 307), which, except for residue 303 and 307, have been reported before (Kida et al., 1987) (see Appendix 8 for sequence alignment, p.270). The difference at amino acid 81 leads to the loss of a glycosylation site for Dk/Hokkaido/33/80 compared to Dk/Ukraine/1/63 and Aichi/2/68. Since Dk/Hokkaido/33/80 contains the same amino acids at positions 137 and 227 as Aichi/2/68, these two viruses differ only at residues 193, 226 and 228 in the RBS. As mentioned previously, Dk/Ukraine/1/63 differs also at residues 137 and 227 from Aichi/2/68.

In order to determine whether the sequence differences between Dk/Hokkaido/33/80 and Dk/Ukraine/1/63 affect the interaction with receptor analogues, receptor-binding studies using SPR were performed for the two viruses. The kinetic rate constants and affinities for α(2,3)- and α(2,6)-fetuin were calculated as described in *Chapter 3* and compared to those of X31 (containing the HA of Aichi/2/68) and its single-site Leu226Gln mutant. As can be seen in Table 11, Dk/Ukraine/1/63 and Dk/Hokkaido/33/80 appear to display very similar receptor-binding properties. This is in agreement with receptor-binding data obtained by means of a solid phase assay (Matrosovich et al., 2000). While the avian viruses preferentially recognise the α(2,3)-linkage, binding to α(2,6)-fetuin was also detected. This result indicates the ability of the pandemic precursor HA to recognise the linkage typical of human isolates, which might have facilitated the transfer from birds to humans. In contrast, no or little interaction with the α(2,6)-linkage was detected in haemagglutination assays (e.g. Rogers and Paulson, 1983; Connor et al., 1994) (see Appendix 3, p.257). Therefore, the sequence differences between the two viruses do not appear to affect their interaction with cellular receptors. The difference in specificity compared to Aichi/2/68 is mainly the result of a ~ 90-fold lower affinity for the α(2,6)-linkage, whereas the binding strength for the α(2,3)-linkage is only ~ 6 times higher. The affinities of Dk/Ukraine/1/63, Dk/Hokkaido/33/80 and Leu226Gln for α(2,3)-fetuin are very similar, whereas the avian viruses bind α(2,6)-fetuin 5-6-fold more weakly than Leu226Gln. This indicates that the differences at positions 193 and 228 between the avian viruses and the Leu226Gln mutant mainly have an effect on affinity for α(2,6)-fetuin.

	α(2,6)-fetuin			α(2,3)-fetuin			
Virus	**10^{-6} x k_a ($M^{-1}s^{-1}$)**	**10^{6} x k_d (s^{-1})**	**10^{14} x K_D (M)**	**10^{-6} x k_a ($M^{-1}s^{-1}$)**	**10^{6} x k_d (s^{-1})**	**10^{14} x K_D (M)**	**Specificity Index (SI)**
Dk/Ukraine/1/63	19.5 ± 3	102 ± 25	523 ± 175	21.2 ± 3	1.8 ± 0.9	8.5 ± 4.8	0.016
Dk/Hokkaido/33/80	28.6 ± 3	120 ± 40	420 ± 150	23 ± 2	1.5 ± 0.6	6.5 ± 2.7	0.015
X31 (Aichi/2/68)	19.2 ± 1	1 ± 0.4	5.2 ± 2	19.2 ± 1	8.5 ± 3	44 ± 15	8.5
Leu226Gln	18.5 ± 2	15.8 ± 3.8	85 ± 21	28.2 ± 2	2 ± 0.5	7.1 ± 2	0.08

Table 11 Kinetic rate constants and affinities of Dk/Ukraine/1/63, Dk/Hokkaido/33/80, X31 (Aichi/2/68 HA) and Leu226Gln viruses for fetuin containing Neu5Ac in the α(2,6)- or α(2,3)-linkage The association rate constants (k_a) and dissociation rate constants (k_d) were determined by SPR. Virus at four different concentrations was injected over the BIAcore sensor chip containing immobilised derivatised fetuins. Kinetic rate constants were determined as described in *Chapter 3* and the affinity expressed as k_d/k_a (K_D). High K_D values indicate low affinity. The specificity index (SI) is K_D (α2,3)/K_D (α2,6) and is a measure for the linkage preference of a virus, with < 1 = preference for α(2,3)- and >1 = preference for α(2,6)-fetuin.

5.3 Setup of the Selection Assay

5.3.1 Selection Procedure Described by Rogers et al. (1985)

Dk/Ukraine/1/63 variants displaying receptor-binding properties typical of human viruses were previously selected by means of RBC derivatised to contain Neu5Ac in the α(2,6)-linkage (Rogers et al., 1985). Virus at titres of 64-128 HAUs was incubated with the human RBC (5% haematocrit) and incubated for 5 min at room temperature. Since Dk/Ukraine/1/63 preferentially recognises α(2,3)-linked Neu5Ac (see above), only variants with the ability to bind to Neu5Ac in the α(2,6)-linkage are expected to bind to these erythrocytes. Upon elution of bound virus from the RBC by incubation with *Clostridium perfringens* sialidase (250 mU/ml) for 3 hours at 37°C, the virus was propagated in MDCK cells to amplify variants. This selection procedure was performed four times followed by plaque purification of potential variants. A total number of 5 of 64 clones were found to be specific for the α(2,6)-linkage, as judged by adsorption to derivatised RBC and inhibition by horse serum, which contains mainly α(2,6)-linked sialic acid. HA sequence analysis revealed that all of the variants contained a single mutation Gln226Leu.

5.3.2 Modification of the Selection Procedure

5.3.2.1 Preparation of RBC to Contain Neu5Ac in the α(2,6)-Linkage

In the assay described above, human RBC were prepared to contain α(2,6)-linked Neu5Ac. This was achieved by treatment of the cells with *Vibrio cholerae* sialidase to remove terminal sialic acid moieties and subsequent resialylation with CMP-Neu5Ac by α(2,6)-linkage specific sialyltransferases purified from rat liver. This enzyme specifically transfers sialic acid to terminal Galβ(1,4)GlcNAc moieties of N-linked glycoproteins. The conditions for resialylation were adjusted to incorporate 35-40 nmoles of Neu5Ac per ml of packed RBC, as judged by adding trace amounts of CMP-[^{14}C]Neu5Ac and measuring

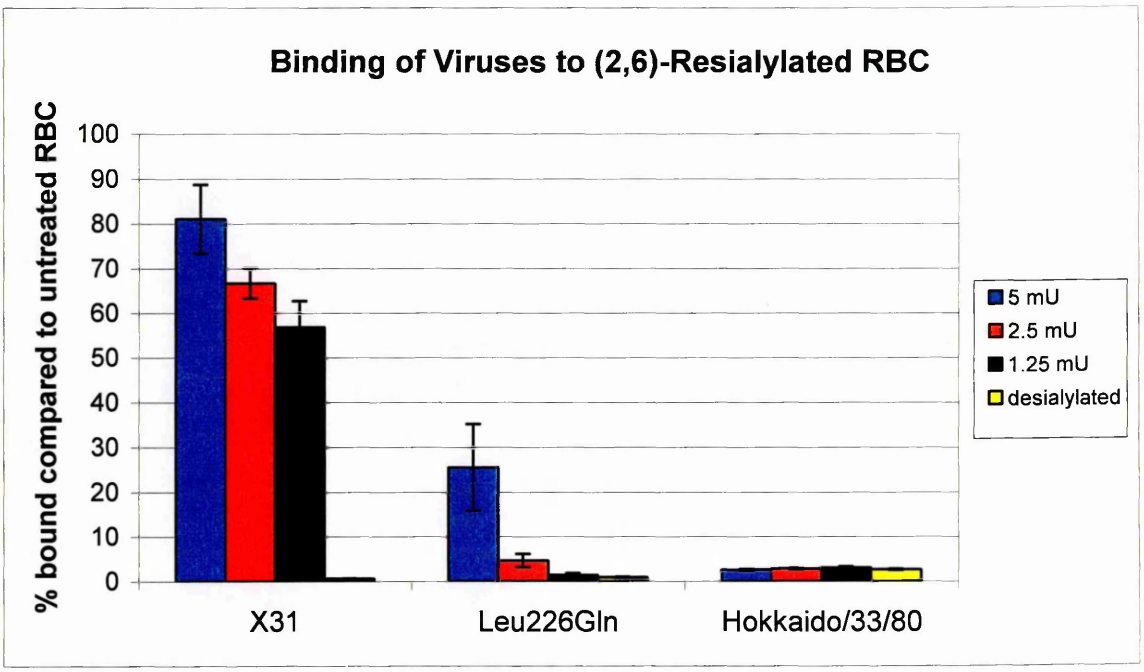
radioactivity. This incorporation level has been shown to be optimal for detecting differences in linkage specificity of influenza viruses in haemagglutination assays (Paulson and Rogers, 1987). In contrast, α(2,3)-resialylated RBC contained higher amounts of Neu5Ac (90-125 nmol/ml packed RBC). The reason for the lower level of Neu5Ac in α(2,6)-reconstituted cells is that higher amounts can lead to recognition of these cells by viruses with α(2,3)-linkage preference, as shown by the Leu226Gln single-site mutant of X31 (Daniels et al., 1987).

Since high selection pressure was considered to be important for the selection of variants, conditions were determined to obtain the highest amount of α(2,6)-linked Neu5Ac on RBS where no binding of the avian viruses was observed. Due to ready availability, turkey RBC instead of human RBC were used for enzymatic treatment. In addition to the de- and resialylation procedure described above, the possibility to selectively remove sialic acid in the α(2,3)-linkage from RBC was also investigated. In contrast to *Vibrio cholerae* NA, which removes sialic acid in the α(2,3)-, α(2,6)- and α(2,8)-linkages (Drzeniek, 1967; Drzeniek and Gauhe, 1970; Schauer, 1982), influenza virus NA of earliest N2 subtype strains introduced into humans in 1957 exhibited clear α(2,3)-linkage preference (Baum and Paulson, 1991). For viruses isolated from 1957-1987, a gradual drift towards cleavage of the α(2,6)-linkage was observed, suggesting co-evolution of HA and NA for preferential recognition of the same receptor determinant. Therefore, RBC were treated with viral sialidases isolated from JAP (Japan/305/57; H2N2) and X31 (Aichi/2/68; H3N2) by bromelain-treatment of virus particles (see *2.2.10 Treatment of Turkey RBC with Viral Neuraminidases, p.86*). De- and resialylation and incubation with the viral sialidases were performed as described in *2.2.11 De- and Resialylation of Turkey RBC, p.86*. Whereas varying amounts of enzyme was used for the resialylation experiment, time was varied for treatment with viral NAs. Then the ability of

X31, its single-site Leu226Gln mutant and Dk/Hokkaido/33/80 to adsorb to the derivatised RBC was tested in a direct binding assay using radioactively labelled viruses. Since Dk/Ukraine/1/63 did not differ in its receptor-binding properties from Dk/Hokkaido/33/80 as determined by SPR, this virus was not included in the experiment. Labelling of the viruses with ^{125}I-Bolton Hunter reagent and the binding experiments were performed as described in *2.2.13 Labelling of Virus with 125Iodine (Bolton-Hunter Reagent), p.87, 2.2.14 Binding Experiments of 125Iodine-labelled Virus to Turkey RBC, p.88.* In summary, the viruses were incubated with the RBC and unbound virus was removed by washing the cells with buffer. Then the amount of virus bound to cells was determined by measuring radioactivity in the RBC sample and expressed as percentage compared to binding to untreated blood.

The results of the binding experiments are presented in Figures 39 and 40. The resialylation experiments show that an increasing amount of α(2,6)-linked Neu5Ac leads to recovery of binding of X31 from less than 1% for desialylated cells up to 80% for resialylated cells without mediating recognition by Dk/Hokkaido/33/80 (Figure 39a). However, increasing amounts of incorporated α(2,6)-linked Neu5Ac led to adsorption of the cells by the α(2,3)-linkage specific Leu226Gln. This finding has been reported before (Daniels et al., 1987) and is most likely the result of a higher affinity of Leu226Gln for receptor analogues containing α(2,6)-linked Neu5Ac compared to Dk/Hokkaido/33/80 as determined by SPR (see Table 11). Therefore, although incubation with the highest amount of enzyme led to the largest ratio of binding of X31 compared to Dk/Hokkaido/33/80 (32:1), binding of X31 to the resialylated blood was only 3-fold higher compared to the Leu226Gln mutant. Since the binding ratio between X31 and Dk/Hokkaido/33/80 was still high upon treatment of the blood with 2.5 mU enzyme (24:1), sialylation under these conditions was considered appropriate for the selection of receptor-

a)



b)

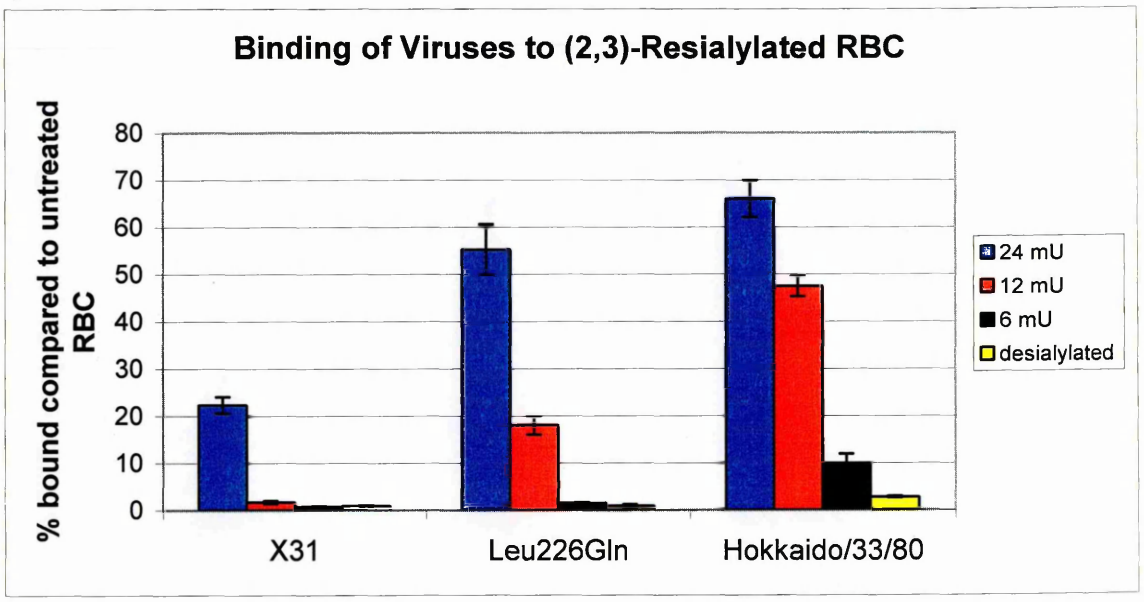
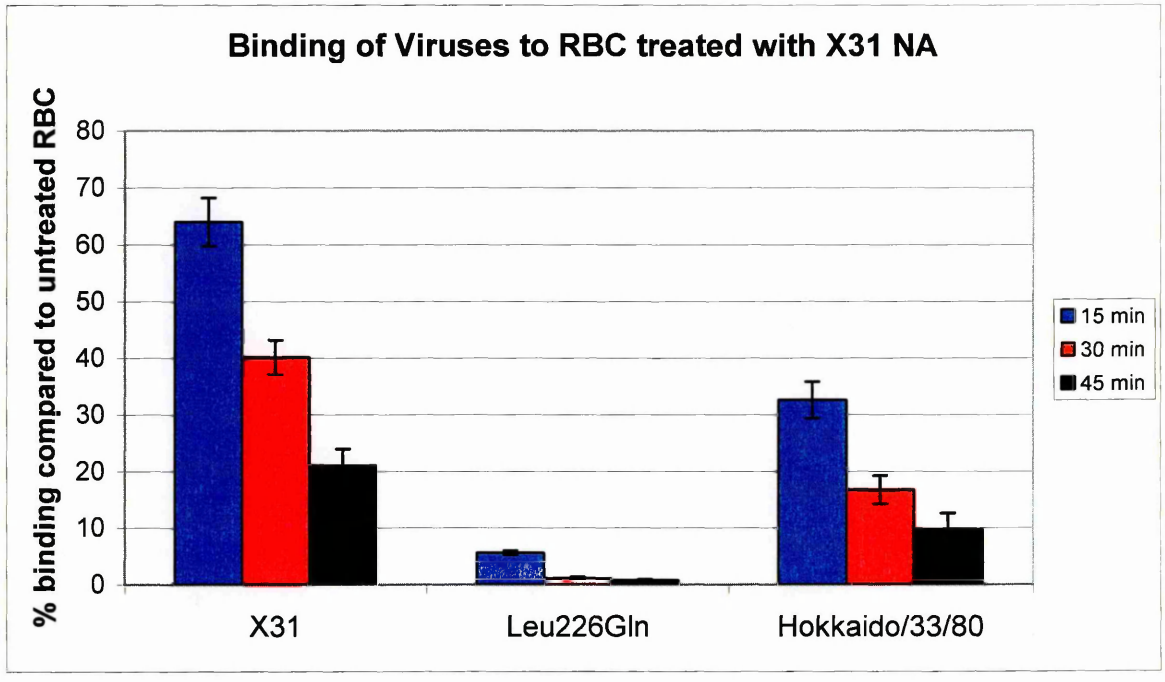


Figure 39 Binding of X31, Leu226Gln and Hokkaido/33/80 viruses to resialylated turkey RBC containing Neu5Ac in the α(2,6)- or α(2,3)-linkage
Sialic acid was removed from RBC by incubation with *Vibrio cholera* sialidase as described in *2.2.11 De- and Resialylation of Turkey RBC, p.86.* Desialylated RBC were reconstituted with Neu5Ac in the α(2,6)-linkage **(a)** or in the α(2,3)-linkage **(b)** by incubation with increasing amounts of linkage-specific sialyltransferases. Binding to the de- and resialylated RBC was assessed by adsorption of ^{125}I-labelled viruses compared to untreated blood.

a)



b)

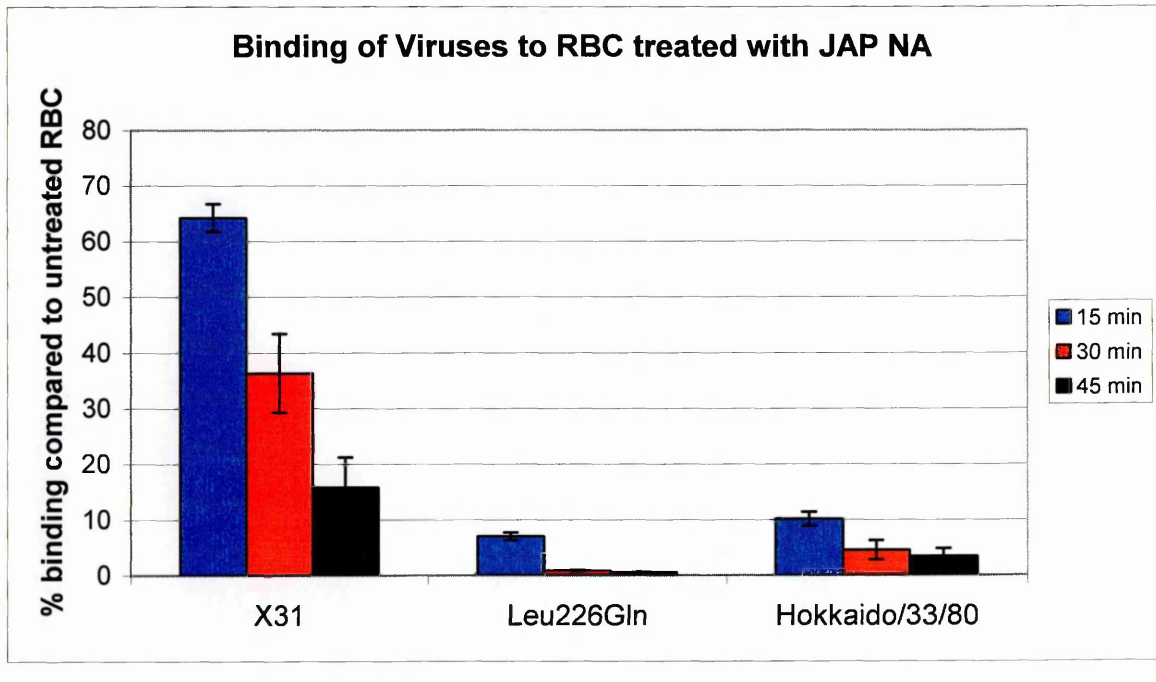


Figure 40 Binding of X31, Leu226Gln and Hokkaido/33/80 viruses to RBC treated with X31 (a) or JAP (b) sialidases for different lengths of time

RBC were treated with viral sialidases as described in *2.2.10 Treatment of Turkey RBC with Viral Neuraminidases, p.86*, in order to remove sialic acid in the α(2,3)-linkage. Binding to the sialidase-treated RBC was assessed by adsorption of ^{125}I-labelled viruses compared to untreated blood.

binding variants. As a control for the resialylation procedure, the effect of incubation of desialylated blood with α(2,3)-sialyltransferase on binding of the viruses was also tested. As expected, binding of α(2,3)-linkage specific Leu226Gln and Dk/Hokkaido/33/80 was recovered upon resialylation with α(2,3)-linked Neu5Ac, (Figure 39b), which is consistent with similar affinities for receptor analogues containing α(2,3)-linked Neu5Ac, as determined by SPR (see Table 11). RBC incubated with the highest amount of enzyme also led to recognition of these cells by X31. This is in agreement with the detected recognition of the α(2,3)-linkage by SPR.

Treatment of RBC with X31 and JAP NA leads to a greater reduction of binding of Dk/Hokkaido/33/80 and Leu226Gln compared to X31, confirming the α(2,3)-linkage preference of these sialidases (see Figures 40a and b). However, substantial decrease in binding could not be gained without a concomitant large reduction of binding by X31. The difference in binding ability of X31 virus compared to the α(2,3)-linkage specific viruses was higher for treatment with JAP compared to X31 NA, in agreement with earlier N2 subtype strains exhibiting higher α(2,3)-linkage specificity compared to viruses isolated in later years (Baum and Paulson, 1991). The maximal ratio of 8:1 (X31:Dk/Hokkaido/33/80) for incubation with JAP NA, however, was 3 times smaller compared to that determined for the de- and resialylation experiments. Therefore, reconstitution of sialic acid depleted RBC by the use of α(2,6)-specific sialyltransferases was chosen for the preparation of RBC for the selection assay.

5.3.2.2 Selection Procedure

The selection procedure was slightly modified from that described by Rogers et al. (1985) and is described in *2.2.12 Selection Experiments, p.87*. Blood no older than four days upon collection from turkeys was de- and resialylated, tested for binding by labelled X31 and

Dk/Hokkaido/33/80 virus and used within 24 hours. Main modifications included the use of different RBC suspensions. Previously, viruses were incubated with RBC at a final concentration of 5% (haematocrit), which the authors considered to represent conditions of large viral excess over cells. In order to increase the selection pressure further, the adsorption was performed at a final concentration of 1% (containing ~ 10^7 cells per ml). In a separate selection process, the viruses were adsorbed to RBC at 0.1, 0.01 and 0.001%, with the aim to reveal the number of cells that would limit the adsorption and hence provide maximal selection pressure. The virus obtained by incubation with the highest dilution of cells was used for the next round of selection. Upon incubation with virus, the blood was washed 6 times instead of 4 times in the presence of BSA in the buffer, in order to decrease non-specific binding of virus to the RBC. Furthermore, the resuspended cells were transferred to a new tube after every wash step, since a negative control consisting of virus added to a tube without blood revealed the ability of virus to stick to the plastic. The selection was performed 7 times compared to 4, in order to increase the probability of accumulation of variants. Whereas adsorption to the 1% RBC suspension readily yielded viruses for every round of selection, the 0.01% suspension represented the highest dilution of blood at which virus was obtained upon propagation in MDCK cells. After the last selection, the viruses selected with 1% and 0.01% RBC suspensions were plaqued on MDCK cells separately. Then 20 clones for every virus (10 for each selection pressure) were analysed for changes in HA_1 amino acid sequence compared to parental virus, which had been propagated the same number of times in MDCK cells. Sequence alignment revealed that none of the clones contained the Gln226Leu change selected previously. The only substitution detected was Arg201Gly for all of the Dk/Hokkaido/33/80 clones compared to parental virus. In contrast, no substitutions were observed for any of the Dk/Ukraine/1/63 viruses.

5.4 Discussion

5.4.1 Inability to Select an Avian HA Containing a Gln226Leu Mutation

A Dk/Ukraine/1/63 variant displaying a shift towards receptor specificity typical of human isolates was previously selected (Rogers et al., 1985). Sequence analysis revealed a mutation Gln226Leu in HA. Since selection in the reverse direction had also been achieved (Rogers et al., 1983a), these results supported the importance of residue 226 for receptor specificity and interspecies transmission. However, in contrast to the single-site Leu226Gln mutant of X31, the mutation reverted back to wild-type upon propagation of the Dk/Ukraine/1/63 variant in eggs. Structural differences in the HAs of Dk/Ukraine/1/63 compared to X31 were suggested to play a role in the genetic instability of the Gln226Leu mutation (Ha et al., 2003). Notably, Pro227 is unique for Dk/Ukraine/1/63, in contrast to other avian and human viruses containing Ser227. Therefore, the selection assay was repeated with an additional potential avian HA progenitor Dk/Hokkaido/33/80, which contains Ser227. Since other residues have been shown to be implicated in defining receptor specificity (see *5.1 Introduction, p.187*), continued selection pressure to recognise Neu5Ac in the α(2,6)-linkage would reveal the importance of other substitutions in this process. Upon selection, the genetic stability of the mutations would then be tested by propagation of these viruses in tissue culture cells and eggs. However, the selection assay described above did not yield any Gln226Leu variants for Dk/Ukraine/1/63 or Dk/Hokkaido/33/80. Whereas none of the analysed Dk/Ukraine/1/63 viruses contained any HA substitutions compared to parental virus, all of the Dk/Hokkaido/33/80 clones contained a mutation Arg201Gly.

The SPR studies show that, while displaying preference for α(2,3)-fetuin, both Dk/Ukraine/1/63 and Dk/Hokkaido/33/80 also have the ability to recognise Neu5Ac in the α(2,6)-linkage (see Table 11). This observation suggests that the selection pressure needed

to obtain a variant with increased affinity for the α(2,6)-linkage would be high. However, as judged by the results obtained in the adsorption assay, the applied selection pressure appeared to be high, since binding of Dk/Ukraine/1/63 to α(2,6)-resialylated cells was as low as for desialylated cells. The conditions for the adsorption experiments and the selection assay were similar with incubation with 1% RBC, since the same amounts of virus and RBC were used.

Although the binding ratio between X31 and Dk/Hokkaido/33/80 for α(2,6)-resialylated blood was high (~ 24:1), a Gln226Leu mutant was not even obtained under conditions considered to be the most stringent (100-fold reduction of the amount of RBC). It therefore appears that the binding strength of the avian viruses to bind to the derivatised blood, although remaining undetected by the adsorption assay, was high enough to recover wild-type virus.

It was assessed whether the failure to obtain the Gln226Leu variant was due to differences in the experimental assays. Whereas the amount of added virus was similar for both selection assays (64-256 HAUs), incubation with RBC was previously performed with RBC at a final concentration of 5%, in contrast to the 1% and 0.01% used here. The use of a 5% suspension and the degree of resialylation were based on a study showing that incubation with X31 at a titre of 4 HAUs leads to maximal binding of 90% compared to the amount bound to native cells (Rogers and Paulson, 1983). In contrast, binding of Dk/Ukraine/1/63 was below 10%. However, the same study showed that when RBC were used as a 1% suspension, the amount of X31 bound was still 70%. It therefore appears unlikely that a concentration difference of RBC of only 5-fold would account for the failure to select the Gln226Leu mutant. Furthermore, the binding ratios of X31 and Dk/Ukraine/1/63 in the adsorption assay described above were similar to those determined in this study. The main difference between the two selection assays was the origin of

erythrocytes. Due to ready availability, turkey RBC instead of human RBC were used in this study. Since the α(2,6)-sialyltransferases in both studies display the same specificities (to transfer sialic acid to terminal Gal of N-linked glycoproteins in the α(2,6)-linkage), other factors such as differences in the density or composition of the glycoproteins on human and turkey RBC might affect the interaction with virus and hence the selection assay. However, since little information on the nature of glycoproteins on turkey RBC is available, the reasons for the inability to obtain the Gln226Leu mutant remain unknown.

5.4.2 Selection of an Arg201Gly Variant

The results of the selection assay indicate the difficulty to select for an avian HA containing the Gln226Leu substitution. Whereas no HA variant was obtained for Dk/Ukraine/1/63, one substitution, Arg201Gly, was detected in all of the 20 clones analysed for Dk/Hokkaido/33/80. The effect of this mutation on receptor-binding properties of this virus has not been determined yet. Residue 201 is located in the HA trimer interface, which is not an obvious location to affect the interaction with cellular receptors. However, it has been shown that an amino acid in the HA interface (218) can influence receptor specificity (Daniels et al., 1987). A virus containing a Gly218Glu was reported to acquire recognition of the α(2,3)-linkage, whereas a Gly218Asp change led to a decrease in affinity for both α(2,3)- and α(2,6)-linked Neu5Ac. It was suggested that the observed change in receptor recognition might have been caused by an altered quaternary structure of HA. Arg201 resides in the opposite side of the RBS to 218 and forms a hydrogen bond to the main-chain carbonyl-group of residue 217 in a neighbouring monomer. Since residue 217 is located next to 218, the substitution at 201 might have an effect on receptor-binding properties. The effect of Gly201 on the interaction with cellular receptors could be assessed by determining the affinity of this variant for α(2,3)- and

α(2,6)-fetuin by SPR.

5.4.3 Possibilities to Improve the Selection Assay Using Turkey RBC

A difficulty in the selection of a receptor-binding variant might be presented by the specificity of the viral NA. As described in *1.10 Functional Balance between HA and NA Activity, p.66*, the activities and specificities of HA and NA need to be carefully balanced for optimal virus replication. Since the HA of Dk/Ukraine/1/63 and Dk/Hokkaido/33/80 was shown to preferentially bind α(2,3)-linked Neu5Ac, the NA is likely to display the same specificity. A variant containing an HA with α(2,6)-linkage specificity but a NA with α(2,3)-linkage specificity might be impaired for efficient replication in MDCK cells and hence be selected against. A drift in NA for α(2,6)-linkage preference might facilitate the selection of an HA variant. Selection pressure directed against NA would be provided by propagation of the virus in cells containing mainly α(2,6)-linked sialic acid. Since human host epithelial cells have been shown to contain mainly α(2,6)-linked sialic acid (Baum and Paulson, 1990; Couceiro et al., 1993), these might have provided the additional selection pressure for efficient selection of an α(2,6)-linkage specific HA variant for the transmission of virus from birds to humans. The routinely used cell lines for propagation of influenza viruses (MDCK and VERO) cells, however, contain both α(2,3)- and α(2,6)-linked sialic acid and would therefore not exert any selection pressure (Govorkova et al., 1996; Ito et al., 1997b). In addition, since human viruses are also exposed to mucins, which contain mainly α(2,3)-linked sialic acid, a combined selection for recognition of α(2,6)-linked sialic acid on host cells but against recognition of α(2,3)-linked sialic acid by mucins might be important for facilitated selection (Breg et al., 1987; Baum and Paulson, 1990; Couceiro et al., 1993). Propagation of virus in MDCK cells in the presence of an α(2,3)-linkage-containing inhibitor might increase the probability of obtaining an HA

variant with increased affinity for α(2,6)-linked sialic acid and concomitant decrease in α(2,3)-linkage-recognition. Alternatively, the selection assay could be repeated with Dk/Ukraine/1/63 and Dk/Hokkaido/33/80 viruses containing an α(2,6)-linkage specific NA generated by reassortment with a human H3 isolate.

6 General Discussion

6.1 Effectiveness of SPR Binding Assay to Study the Interaction of Influenza Virus with Receptor Analogues

A receptor-binding assay using the BIAcore has been set-up to study the interaction of influenza viruses with receptor analogues. This method makes use of the optical phenomenon of SPR, a technique widely used to study receptor-ligand specificity, affinity and kinetic rate constants for many biomolecular interactions, including viral proteins and whole virus particles (e.g. Casasnovas and Springer, 1995; McDermott et al., 2000). This assay has previously been used to study the kinetics and affinity of BHA-rosettes derived from influenza virus for a sialic acid-containing receptor analogue, fetuin (Takemoto et al., 1996). The data presented here show that SPR assays can be performed with whole virus particles. In addition, the method was further developed to directly compare the affinity of virus for fetuin containing Neu5Ac either in the human-specific (α(2,6)) or the avian-specific (α(2,3)) linkage, allowing for the study of linkage specificity. The K_D was determined from the kinetic rate constants, since steady-state conditions were not reached on the time-scale of the experiments. The SPR assay allowed for the detection of a broad range of affinity differences, from ~ 3-fold, indicative of high sensitivity, up to ~ 3,000-fold. Furthermore, the experiments were highly reproducible with different batches of virus.

The affinity of virus particles for fetuin and derivatised fetuin was revealed to be very high, e.g. the K_D of the interaction of the laboratory strain X31 for fetuin was ~ 10^{-13} M. This interaction is ten orders of magnitude tighter compared to that of BHA or virus and trisaccharides (K_D of ~ 10^{-3} M) (Sauter et al., 1989; Hanson et al., 1992). This affinity difference is accounted for by the simultaneous binding of multiple HA trimers on the

virus envelope to multiple Neu5Ac on fetuin (multivalency), leading to amplification of the 1:1 (monovalency) binding affinity. The virus-fetuin affinity is also five orders of magnitude higher compared to the BHA rosettes-fetuin affinity previously determined by SPR ($K_D \sim 10^{-7}$ M) (Takemoto et al., 1996). This increase in affinity might also be explained by an increase in valency, since the virus envelope contains ~ 500 HA trimers compared to ~ 6-10 per BHA rosette. The advantage of the high affinity of the virus-fetuin interaction is that only small amounts of virus are needed (in the range of the K_D) for reliable measurements. The highest amount of virus used in the SPR assay was 0.26 nM (~ 15 μg/ml total viral protein), in contrast to BHA rosettes, which had previously been used at a concentration of 120 nM (~ 200 μg/ml HA). Furthermore, clear determination of the K_D for the BHA rosette-fetuin interaction proved unsuccessful in this study due to the high heterogeneity observed in the reaction curves. This is likely to be the result of a heterogeneous rosette population with regard to the number of BHA trimers per rosette and disagrees with the reported suitability of rosettes for affinity measurements by SPR. Visual inspection of the reaction curves of the BHA rosette-fetuin interaction studied by Takemoto et al. (1996) also suggests heterogeneity. Since little information on the data analysis is provided, it is not clear how this heterogeneity was taken into account in the calculation of the K_D.

A number of receptor-binding assays are available to study the interaction of influenza virus with receptor analogues. However, quantitative binding data has so far only been obtained by NMR, fluorescence polarisation and solid-phase assays (Gambaryan and Matrosovich, 1992; Hanson et al., 1992; Sauter et al., 1992; Weinhold and Knowles, 1992). Of these, the solid-phase assay has been used to routinely detect affinity and specificity differences between influenza viruses. This method determines equilibrium dissociation constants indirectly by measuring the amount of HRP-labelled fetuin bound to

virus adsorbed to 96-well microtitre plates in the presence of different amounts of the receptor analogue of interest. The advantage of this assay is that many viruses can be tested simultaneously. In contrast, the SPR method is more time-consuming, since the dataset for every virus has to be collected separately, which takes about 3 hours. Therefore, BIAcore is not a suitable method for high-throughput analysis. However, programming of the injection commands allows the experiments to be performed automatically without the need for attendance. In addition, only small amounts of derivatised fetuin are required for coupling to the sensor chip (~ 2 μg). Since fetuin is very resistant with regard to removal of bound virus by regeneration buffers, it is possible to re-use the same sensor surface for many binding experiments (> 100 times). In contrast, determination of the K_D in solid phase assays requires higher amounts of receptor analogues, since they need to be used at range of concentrations and can not be re-used. Since concentrations of analytes range from mM (sialyllactose) to μM (sialyllactose attached to polyacrylic acid carriers), the higher consumption of receptor analogues renders the solid-phase assay less cost-effective. A further advantage of the established SPR assay is that absolute affinities can be compared for different viruses, due to the controlled concentrations of both ligand and analyte. In contrast, absolute affinity comparison is more difficult for solid-phase assays, where virus is bound to 96-well microtitre plates either directly by physical adsorption or indirectly to fetuin-coated wells. The amount of adsorbed virus might therefore vary with the absolute affinity of the viruses, especially when these are adsorbed to fetuin-coated wells.

Furthermore, the SPR assay also gives information on the kinetic rate constants k_a and k_d for the virus-fetuin interaction, thereby providing insight into which rate is the major determinant for binding strength.

In conclusion, despite being time-consuming, the development of the SPR assay

has generated an effective method to routinely study affinities of influenza virus for receptor analogues.

6.2 Changes in Receptor-Binding Properties in Relation to Antigenic Drift

The established SPR assay was used to study the receptor-binding properties of a panel of H1 and H3 viruses. These were chosen on the basis of differences in their antigenicity, as determined by HI tests with post-infection ferret sera. Since HA undergoes continuous antigenic drift in order to evade pre-existing immunity in the human population, it was of interest to investigate whether changes in antigenicity would correlate with differences in their affinity for receptor analogues containing α(2,3)- or α(2,6)-linked Neu5Ac. An interrelationship between these two properties is thought to be due to the location of antigenic sites in close vicinity of the RBS (see *1.7.6 Interrelationship between Antigenic Variation and Receptor-Binding Properties, p.58*). Furthermore, substitutions in antigenic sites further away from the RBS can affect the interaction with cellular receptors if they create or abolish carbohydrate attachment sites.

The data presented in this study show that evolution of H1 and H3 viruses in the human population is not only associated with antigenic variation but also with changes in affinities for receptor analogues. Notably, the emergence of antigenic variants was accompanied by distinct changes in receptor-binding properties for the H3 viruses since 2002 (Fujian-like viruses) and for the H1 viruses since 1999 (New Caledonia-like viruses). The Fujian-like viruses were characterised by a large decrease in affinity for α(2,3)-fetuin, resulting in a large increase in α(2,6)-linkage preference. The New Caledonia-like viruses also displayed clear α(2,6)-linkage specificity, in contrast to the remaining H1 isolates, which were either α(2,3)- or "dual"-linkage specific. The differences in both antigenic and

receptor-binding properties were correlated with many amino acid substitutions close to or in the RBS. It has initially been thought that RBS residues would have to be conserved in the process of antigenic drift in order to preserve receptor-binding function (Both et al., 1983b; Weis et al., 1988; Bizebard et al., 1995). However, the observed changes at RBS residues, in agreement with earlier studies (e.g. Lindstrom et al., 1996; Mori et al., 1999; Nobusawa et al., 2000), strongly indicate that the plasticity of the RBS to accommodate cellular receptors is higher than previously thought. It should be noted that antigenic changes did not always result in large differences in affinity for receptor analogues. For example, Johannesburg/33/94 and Sydney/5/94 (H3 viruses), and Brazil/11/78 and Chile/1/83 (H1 viruses) were antigenically distinct but displayed similar receptor-binding properties. This finding suggests that residues that are involved in recognition by antibodies differ in their propensity to affect receptor-binding properties.

The changes at residues close to the RBS are likely to be involved in antigenic drift, based on the observation that many of these are located in previously defined antigenic sites and the surface-exposed nature of their side-chains.

The main driving force for HA evolution is likely to be escape from pre-existing immunity, so as to maintain infectivity of the virus. Therefore, the substitutions in the RBS could be a consequence of immune pressure as well. These could play a direct role in escape from neutralising antibodies if they are part of an antibody-combining site. On the other hand, their involvement could be of indirect character, either to compensate negative effects on receptor function caused by a change in an antigenic site affecting the structure of the RBS or by modulation of receptor-binding properties as an alternative mechanism for antibody-escape. Since binding to antibody is in direct competition with attachment to cells, it has been suggested that an increase in affinity for cellular receptors might be sufficient to prevent neutralisation by antibodies. This hypothesis is based on the

observation that adsorptive mutants are often selected with low-affinity antibodies or when antibodies are provided at sub-neutralising concentrations (Fazekas de St. Groth, 1977; Yewdell et al., 1986; Temoltzin-Palacios and Thomas, 1994; Laeeq et al., 1997). Since concomitant immunity to previous infection has been suggested to lead to the production of antibodies with reduced affinity (Fazekas de St. Groth and Webster, 1966), it is possible that such an alternative mechanism for immune-evasion operates in the human host.

Interestingly, the Fujian-like viruses were shown to generally display a lower affinity for α(2,6)-fetuin compared to earlier isolates, which would be expected to have a negative effect on binding to human receptors. It is intriguing to speculate that the large reduction in affinity for α(2,3)-linked Neu5Ac would facilitate access of these viruses to cells by preventing binding to inhibitory mucins, which contain mainly α(2,3)-linked sialic acid, in the respiratory tract, thereby compensating for the negative effect of reduced binding strength for human receptors.

6.3 Implication of Changes in Receptor-Binding Properties for Virus Infectivity

Changes in receptor-binding properties as a direct or indirect result of immune pressure are expected to have implications for the fitness of influenza virus. Although escape from immune surveillance is essential for re-infection of the human population, receptor-binding function is also important for virus infectivity. Therefore, a certain balance between the ability to escape from neutralising antibodies and the ability to bind to cells needs to be maintained. An increase in affinity as an alternative mechanism to antigenic drift would undoubtedly provide an evolutionary advantage for a virus and would therefore be selected. However, since mutations are by definition not directed, many substitutions might be detrimental instead of beneficial for virus fitness. It has therefore been proposed

that the rapid rate of antigenic drift might eventually impair the virus' ability to survive in the human host due to the accumulation of detrimental point mutations (Fitch et al., 1991), a concept referred to as Muller's ratchet (Muller, 1932; Felsenstein, 1974). Since HA appears to be in evolutionary stasis in aquatic birds (Kida et al., 1987; Gorman et al., 1990a; Gorman et al., 1990b; Bean et al., 1992), it has been suggested that replacement of a deleterious HA with an HA from the avian reservoir by reassortment would restore the fitness of influenza virus (Fitch et al., 1991). Such a mechanism would imply the necessity for the introduction of an avian HA for the maintenance of influenza virus in the human host. Although Muller's ratchet is an intriguing explanation for the emergence of pandemic strains, evidence for such a mechanism has not been provided (Donis, 1991). Alternatively, it is conceivable that the number of progeny virus is simply high enough in order to select against variants with deleterious mutations in a "quasi-species" population (Fitch et al., 1991; Domingo et al., 2001). Four of the five tested recent Fujian-like viruses displayed a lower absolute affinity for both α(2,3)- and α(2,6)-fetuin compared to earlier viruses. However, receptor-binding data on many more isolates would be needed to determine whether a low affinity would be characteristic of current H3 viruses.

6.4 Significance of Receptor-Binding Data Derived from Egg-Adapted Viruses

The phenomenon of host cell-mediated variation has been shown to occur when clinical isolates are propagated in the laboratory, especially in eggs (see *4.1.3 Host Cell-Mediated Variation, p.134*). This process needs to be taken into account when receptor-binding properties of viruses are studied, since they might not be representative of the original clinical isolates. The viruses used in this study were all egg-adapted and their HA_1 sequence of the original clinical isolate was not readily available. Therefore, it is not clear

how well the determined antigenic and receptor-binding properties reflect the characteristics of naturally circulating viruses. However, it was the aim of this study to investigate if antigenically distinct viruses would also display differences in their receptor-binding properties, irrespective of whether they reflect those of the original isolate. Nevertheless, sequencing of HA directly from the clinical specimen before propagation in the laboratory and cultivation of virus in MDCK or VERO cells, where host cell-mediated variation is less likely to occur, would enable to correlate these data to field strains. This might lead to a better understanding of evolution of antigenic and receptor-binding properties in the human host.

6.5 Implications of Changes in Receptor-Binding Properties for Surveillance Studies

The decision for including a novel strain in the influenza vaccine formulation is based on detection of antigenic variation based on HI tests using post-infection ferret sera. This assay reflects the conditions in the human respiratory tract, since antibody and cells compete for the binding to virus. However, a major drawback of this competition-based method is that it is significantly affected by changes in receptor-binding properties of a virus, which complicates the analysis of HI tests. For example, a virus with increased affinity for cellular receptors requires a higher amount of antibody to inhibit binding to RBC. As a result, such a virus might be classified as antigenically different, unless antibodies are provided at higher concentrations, which also presents technical difficulties in the process of antibody production. In contrast, binding to RBC of a virus with low affinity for cellular receptors would be inhibited by very low concentrations of antibody. An indication of low-affinity viruses is that they often give higher titres with heterologous ferret serum than the virus probed with its homologous serum. Therefore, the amount of

antibody needs to be adjusted depending on the receptor-binding properties of a particular strains, which makes quantitation of reactivity with antibodies more difficult. Furthermore, the HI test can be performed with RBC of different origins, human, guinea pig, chicken or turkey (WHO, 2005c). Little information on the carbohydrate composition is available for turkey RBC, whereas the remaining RBC have been shown to differ in the relative prevalence of sialic acid in the α(2,3)- versus the α(2,6)-linkage (Ito et al., 1997a; Medeiros et al., 2001). Changes in affinity for an individual virus for only one of these linkages might therefore affect the extent to which the HI is affected depending on the nature of RBC used. Although these problems can be resolved by the use of assays that are not affected by receptor-binding properties, such as immunodiffusion and ELISA, these methods are more labour-intense.

Efforts are currently being made to quantify the antigenic relationships between viruses based on HI tests by applying mathematical models for the analysis of the results (Smith et al., 2004). These allow for an increase in sensitivity of measurements of antigenic differences and might help in the comparison of HI data from different laboratories. Information on receptor-binding properties might provide valuable data to be included in such mathematical models for further improvement in the analysis of antigenic data, prediction of antigenic evolution and vaccine strain selection.

6.6 Future Experiments

6.6.1 Modification of the SPR Assay for the Use of Virus Propagated in Mammalian Tissue Culture Cells

The SPR assay was set-up with egg-adapted viruses. The rationale for the use of eggs was that growth in this host cell system yields a homogeneous population with regard to shape and size of virus particles. This was considered important to avoid heterogeneity in the

association and dissociation curves in SPR studies, which would complicate the analysis of kinetics data. In addition, once adapted, viruses usually grow to high titres in eggs. However, the Fujian-like H3 viruses grew poorly in this host cell system, probably due to their decrease in affinity for α(2,3)-linked sialic acid, the predominant linkage in cells of the allantoic cavity (Ito et al., 1997b). Since growth restriction increases the chance of host cell-mediated variation, it would be of advantage to propagate these viruses in mammalian tissue culture cells, such as MDCK or VERO cells. These have been shown to select for variants less frequently and the antigenic and receptor-binding properties of viruses cultivated in such cells would therefore more closely reflect those of the respective clinical isolates. In addition, eggs present additional drawbacks, such as the lack of year-round supplies of high quality eggs and the possible presence of pathogens. Therefore, it would be of interest to propagate viruses in mammalian tissue culture cells for both antigenic and SPR studies. The observation that tissue culture isolates are usually more heterogeneous in particle size and shape compared to egg-isolates could potentially complicate analysis of kinetics data. However, if necessary, the virus purification method could be adjusted for the separation of different sized particles, for example by collecting narrower bands in the density centrifugation.

6.6.2 Testing Additional Isolates to Confirm Distinct Receptor-Binding Properties of Recent H3 and H1 Viruses

The SPR data suggest that the distinct receptor-binding properties of the Fujian-like viruses (large decrease in affinity for α(2,3)-fetuin) and New Caledonia-like viruses (α(2,6)-linkage specificity) would be characteristic of viruses circulating since 2002 and 1999, respectively. In order to confirm this finding, additional isolates would have to be tested, possibly grown in mammalian tissue culture cells (for reasons stated above).

6.6.3 Use of Reverse Genetics for Generation of Mutant Influenza Viruses

The generation of influenza virus entirely from cDNA (reverse genetics) has become a powerful tool for the generation of mutant influenza viruses (Neumann et al., 1999; Hoffmann et al., 2000). This technique could be applied for the investigation of the subjects described below.

6.6.3.1 Assessment of the Structural Basis for Reduction in Affinity for α(2,3)-fetuin of Fujian-like Viruses

The data presented in this study suggests that the reduced affinity of Fujian-like viruses for α(2,3)-fetuin is a result of substitutions at HA residues 222 and 225. These might potentially relocate an oligosaccharide protruding from residue 165 that would interfere with distant parts of receptors containing α(2,3)-linked sialic acid. X-ray studies of HAs of these viruses and the single-site mutant Gly225Asp complexed with receptor analogues might provide a structural basis for this observation. Furthermore, the involvement of a carbohydrate moiety in steric hindrance would be addressed by structural and SPR studies with a single-site mutant that lacks this glycosylation site.

6.6.3.2 Assessment of the Structural Basis for Residues Involved in Linkage-Recognition for H1 Isolates

The structures of Puerto Rico/8/34 and Sw/Iowa/15/30 HAs complexed with receptor analogues have provided first insight into residues involved in linkage specificity for the H1 viruses (Gamblin et al., 2004). The SPR data presented in this study proposes that linkage-recognition is defined by a combination of amino acids in the RBS. X-ray studies with a number of viruses used in this study would address the roles of these residues in linkage specificity. As for the H3 viruses, the importance of individual amino acids in

distinguishing between receptor analogues containing Neu5Ac in the α(2,3)- or α(2,6)-linkage would be assessed by receptor-binding studies using single-site mutants.

6.6.3.3 Residues Responsible for Distinct Receptor-Binding Properties of H1N2 Virus

All the H1 isolates of 1999-2003 (New Caledonia-like) have been shown to display very similar receptor-binding properties, with the exception of the H1N2 virus, which differed from the others by a 10-fold increase in affinity for α(2,6)-fetuin and a 100-fold decrease in affinity for α(2,3)-fetuin. Since Egypt/96/02 was the only reassortant virus available for this study, it is not known whether these receptor-binding properties are typical of H1N2 viruses in general. Therefore, additional reassortant viruses would have to be tested for their affinity for α(2,3)- and α(2,6)-fetuin. In addition, since Egypt/96/02 differs from the remaining New Caledonia-like viruses only at residues 132, 193 and 218, it would be of interest to elucidate the roles of these in linkage-recognition by X-ray studies and by testing receptor-binding properties of single-site mutants.

6.6.4 Studies of NA Activity and Specificity

This study only addressed receptor-binding properties of influenza virus. Since evidence for a functional interplay between HA and NA for optimal virus replication has been provided by a number of studies (see *1.10 Functional Balance between HA and NA Activity, p.66*), it would be of interest to complement the receptor-binding data with NA activity and specificity data. Activity would be tested in a routinely performed fluorometric assay using labelled sialic acid (muNANA assay) (Potier et al., 1979), whereas specificity studies would require labelled receptor analogues containing α(2,6)- or α(2,3)-linked Neu5Ac (e.g. Mochalova et al., 2005). NA studies would be of particular importance for recent H1, H3 viruses and the H1N2 virus Egypt/96/02. Notably, the H1N2

isolate displayed a similar low affinity for α(2,3)-fetuin as the recent H3 viruses. Since it has previously been suggested that substitutions in H1 HA accumulated from 1995-1999 might have facilitated the reassortment between H1N1 and H3N2 viruses, these studies would further elucidate the importance of a functional balance between HA and NA.

6.6.5 Investigation of Involvement of RBS Residues in Antigenic Variation

The performed studies did not assess whether the observed changes for H1 and H3 isolates in the RBS could be directly involved in antigenic drift by blocking binding to antibodies, or whether they merely affected receptor-binding properties. ELISA studies with an extensive panel of mAb of known epitope specificity would address this question. Furthermore, variants could be selected with mAb generated against individual isolates. Substitutions at RBS residues that would lead to a large decrease in binding to the selecting mAb in ELISA tests would provide evidence for the involvement of these residues in antibody-binding.

6.6.6 Testing of Receptor-Binding Properties of the Selection Assay Variant

The attempt to repeat the selection of an avian H3 virus containing a Gln226Leu substitution (Rogers et al., 1985) did not prove successful. However, since an Arg201Gly variant was selected for Dk/Hokkaido/33/80 instead, it would be of interest to test the effect of this single-site substitution on linkage-recognition.

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8 Appendix

Amino acid	Three letter code	One letter code
alanine	ala	A
arginine	arg	R
asparagine	asn	N
aspartic acid	asp	D
asparagine or aspartic acid	asx	B
cysteine	cys	C
glutamic acid	glu	E
glutamine	gln	Q
glutamine or glutamic acid	glx	Z
glycine	gly	G
histidine	his	H
isoleucine	ile	I
leucine	leu	L
lysine	lys	K
methionine	met	M
phenylalanine	phe	F
proline	pro	P
serine	ser	S
threonine	thr	T
tryptophan	try	W
tyrosine	tyr	Y
valine	val	V

Appendix 1 Amino acids, one- and three-letter codes

A homogeneous 1:1 interaction between analyte and ligand is described by the equation

$$A + B \underset{k_d}{\overset{k_a}{\rightleftharpoons}} AB$$

where A is the analyte, B the surface-bound ligand, k_a the association rate constant and k_d the dissociation rate constant. The rate of complex formation is written as

$$\frac{d[AB]}{dt} = k_a[A][B] - k_d[AB]$$

The concentration of free ligand [B] is the difference between the total amount of ligand $[B]_0$ and the amount of complex

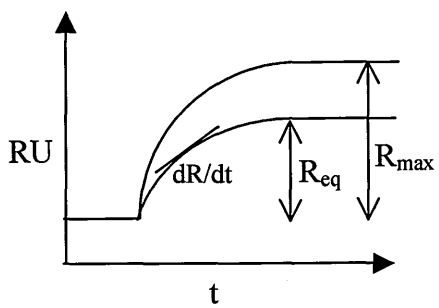
$$[B] = [B]_0 - [AB]$$

Substitution in the rate equation for complex formation gives

$$\frac{d[AB]}{dt} = k_a[A]([B]_0 - [AB]) - k_d[AB]$$

In terms of the SPR signal, this equation is written as

$$\frac{dR}{dt} = k_a C(R_{max} - R) - k_d R$$



where dR/dt is the rate of change of the SPR signal, C the concentration of free analyte A, R_{max} the maximum analyte binding capacity in RU and R the SPR signal in RU at time t. Since sample injection is continuous, the concentration of free analyte C equals the concentration of analyte in the sample.

The equation can be rearranged to

$$\frac{dR}{dt} = k_a C R_{max} - (k_a C + k_d)R$$

Integration of this equation gives

$$R(t) = \frac{k_a C R_{max}}{k_a C + k_d}\left(1 - e^{-(k_a C + k_d)t}\right) = R_{eq}(1 - e^{-k_{obs}t})$$

with $k_{obs} = k_a C + k_d$ and $R_{eq} = \frac{k_a C R_{max}}{k_a C + k_d}$

Appendix 2 Derivation of association kinetics equation for SPR assays

Appendix 3 Receptor-binding data found in literature on H1 and H3 viruses used in this study

3'SL = Neu5Acα(2,3)Galβ1,4Glc

6'SL = Neu5Acα(2,6)Galβ1,4Glc

6'SLN = Neu5Acα(2,6)Galβ1,4GlcNAc

paraglobosides IV^3 (NeuAc)nLc4Cer) = Neu5Acα(2,3)Galβ1,4GlcNAcβ1,3Galβ1,4Glcβ1-Ceramide

IV^6 (NeuAc)nLc4Cer) = Neu5Acα(2,6)Galβ1,4GlcNAcβ1,3Galβ1,4Glcβ1-Ceramide

PAA = polyacrylamide-carrier (for use of polyvalent receptor analogues)

α(2,6)-linkage	α(2,3)-linkage	Reference	Assay/receptor analogues used
X31			
85	49	(Nobusawa et al., 1991)	absorbance at 540 nm of virus-mediated haemolysis (super-natant) of paragloboside -coated RBC–absorbance of haemolysis of desialylated RBC/pmol ganglioside attached to 10^6 RBC
+++	++	(Masuda et al., 1999)	TLC with paraglobosides (intensity of binding)
3%	1%	(Ryan-Poirier et al., 1998)	TLC with paraglobosides (percentage of total intensity of binding, 100% includes other receptor-analogues as well)
128/256/1024	0	(Rogers and Paulson, 1983)	HAU, haemagglutination assay (resialylated RBC)
4096	0	(Rogers et al., 1983a)	HAU, haemagglutination assay (resialylated RBC)
64	0	(Rogers and D'Souza, 1989)	HAU, haemagglutination assay (resialylated RBC)
4096	0	(Connor et al., 1994)	HAU, haemagglutination assay (resialylated RBC)
1024	0	(Anders et al., 1986)	HAU, haemagglutination assay (resialylated RBC)
1024	0	(Daniels et al., 1987)	HAU, haemagglutination assay (resialylated RBC)
13	>142	(Daniels et al., 1987)	adsorption assay, amount of sialic acid to bind 50% virus (nmol/ml packed RBC)
128	32	(Suzuki et al., 1986)	HAU, haemagglutination assay (RBC reconstituted with paraglobosides)
128	32	(Suzuki et al., 1987)	HAU, haemagglutination assay (RBC reconsituted with resialylated glycoprotein 2)

α(2,6)-linkage	α(2,3)-linkage	Reference	Assay/receptor analogues used
X31			
1 mM	2 mM	(Matrosovich et al., 1997)	K_D, solid phase assay, 3'SL, 6'SLN
0.1 μM	0.5 μM	(Matrosovich et al., 2000)	K_D, solid phase assay, 3'SL-, 6'SLN-PAA
2.5 mM	6.3 mM	(Gambaryan and Matrosovich, 1992)	K_D, solid phase assay, 3'SL, 6'SL
4 mM	> 10mM	(Matrosovich et al., 1993)	K_D, solid phase assay, 3'SL, 6'SLN
2.1 mM	3.2 mM	(Sauter et al., 1989)	K_D, NMR with BHA, 3'SL, 6'SL
2.7 mM	3.5 mM	(Hanson et al., 1992)	K_D, NMR with virus, 3'SL, 6'SL
Leu226Gln			
0	1024	(Anders et al., 1986)	HAU, haemagglutination assay (resialylated RBC)
0	4096	(Rogers et al., 1983a)	HAU, haemagglutination assay (resialylated RBC)
0	16	(Rogers and D'Souza, 1989)	HAU, haemagglutination assay (resialylated RBC)
0	1024	(Daniels et al., 1987)	HAU, haemagglutination assay (resialylated RBC)
53	46	(Daniels et al., 1987)	adsorption assay, amount of sialic acid to bind 50% virus (nmol/ml packed RBC)
> 10mM	3.1 mM	(Matrosovich et al., 1993)	K_D, solid phase assay, 3'SL, 6'SL
3 mM	1 mM	(Matrosovich et al., 1997)	K_D, solid phase assay, 3'SL, 6'SLN
5.9 mM	2.9 mM	(Sauter et al., 1989)	K_D, NMR with BHA, 3'SL, 6'SL

Appendix 3 (continued)

α(2,6)-linkage	α(2,3)-linkage	Reference	Assay/receptor analogues used
Sydney/5/97			
128	64	(Medeiros et al., 2001)	HAU, haemagglutination assay (resialylated RBC)
Dk/Ukraine/1/63			
0.67μM	0.04μM	(Matrosovich et al., 2000)	K_D, solid phase assay, 3'SL-, 6'SLN-PAA
>100μM (>100μM)	40μM	(Gambaryan et al., 1997)	K_D, solid phase assay, 3'SL-, 6'SLN-PAA (6'SL-PAA)
2.5mM	0.025mM	(Gambaryan and Matrosovich,1992)	K_D, solid phase assay, 3'SL, 6'SL
1.25mM (2.5mM)	0.02mM	(Matrosovich et al., 1993)	K_D, solid phase assay, 3'SL, 6'SLN (6'SL)
>2mM	0.4mM	(Matrosovich et al., 1997)	K_D, solid phase assay, 3'SL, 6'SL
53	78	(Nobusawa et al., 1991)	absorbance at 540 nm of virus-mediated haemolysis (super-natant) of paragloboside -coated RBC–absorbance of haemolysis of desialylated RBC/pmol ganglioside attached to 10^6 RBC
0	4096	(Connor et al., 1994)	HAU, haemagglutination assay (resialyated RBC)
8	256	(Rogers and Paulson, 1983)	HAU, haemagglutination assay (resialyated RBC)
0	64	(Rogers et al., 1985)	HAU, haemagglutination assay (resialyated RBC)
Dk/Hokkaido/33/80			
0.33 μM	0.02 μM	(Matrosovich et al., 2000)	K_D, solid phase assay, 3'SL-, 6'SLN-PAA

Appendix 3(continued)

α(2,6)-linkage	α(2,3)-linkage	Reference	Assay/receptor analogues used
Puerto Rico/8/34			
2048	1024	(Medeiros et al., 2001)	HAU, haemagglutination assay (resialyated RBC)
128	256	(Rogers and Paulson, 1983)	HAU, haemagglutination assay (resialyated RBC)
+	+++	(Suzuki et al., 1992)	TLC with paraglobosides (intensity of binding)
32	128	(Suzuki et al., 1987)	HAU, haemagglutination assay (RBC reconstituted with 3/6' glycoprotein 2)
64	128	(Suzuki et al., 1986)	HAU, haemagglutination assay (RBC reconstituted with paragloboside)
128	512	(Rogers and D'Souza, 1989)	HAU, haemagglutination assay (resialyated RBC)
107%	107%	(Rogers and D'Souza, 1989)	percentage bound compared to native RBC, adsorption assay (resialylated RBC)
0.03mM (0.5mM)	0.63mM	(Matrosovich et al., 1993)	K_D, solid phase assay, 3'SL, 6'SLN (6'SL)
Sw/Iowa/15/30			
1024	256	(Rogers and D'Souza, 1989)	HAU, haemagglutination assay (resialyated RBC)
108%	77%	(Rogers and D'Souza, 1989)	percentage bound compared to native RBC adsorption assay (resialylated RBC)
8	0	(Ito et al., 1998)	HAU, haemagglutination assay (resialyated RBC)

Appendix 3 (continued)

α(2,6)-linkage	α(2,3)-linkage	Reference	Assay/receptor analogues used
Dk/Alberta/35/76			
1μM	0.014μM	(Matrosovich et al., 2000)	K_D, solid phase assay, 3'SL-, 6'SLN-PAA
>100μM (>100μM)	30μM	(Gambaryan et al., 1997)	K_D, solid phase assay, 3'SL-, 6'SLN-PAA (6'SL-PAA)
0	2048	(Ito et al., 1998)	HAU, haemagglutination assay (resialyated RBC)
>2mM	0.05mM	(Matrosovich et al., 1997)	K_D, solid phase assay 3'SL, 6'SL
0	2048	(Rogers and D'Souza, 1989)	HAU, haemagglutination assay (resialyated RBC)
0%	100%	(Rogers and D'Souza, 1989)	percentage bound compared to native RBC adsorption assay (resialylated RBC)
Fort Monmouth/1/47			
256	512	(Rogers and Paulson, 1983)	HAU, haemagglutination assay (resialyated RBC)
1024	128	(Rogers and D'Souza, 1989)	HAU, haemagglutination assay (resialyated RBC)
113%	89%	(Rogers and D'Souza, 1989)	percentage bound compared to native RBC, adsorption assay (resialylated RBC)
Fort Warren/1/50			
0.12mM (1.6mM)	0.1mM	(Matrosovich et al., 1993)	K_D, solid phase assay 3'SL, 6'SLN (6'SL)
1024	128	(Rogers and D'Souza, 1989)	HAU, haemagglutination assay (resialyated RBC)
82%	106%	(Rogers and D'Souza, 1989)	percentage bound compared to native RBC, adsorption assay (resialylated RBC)

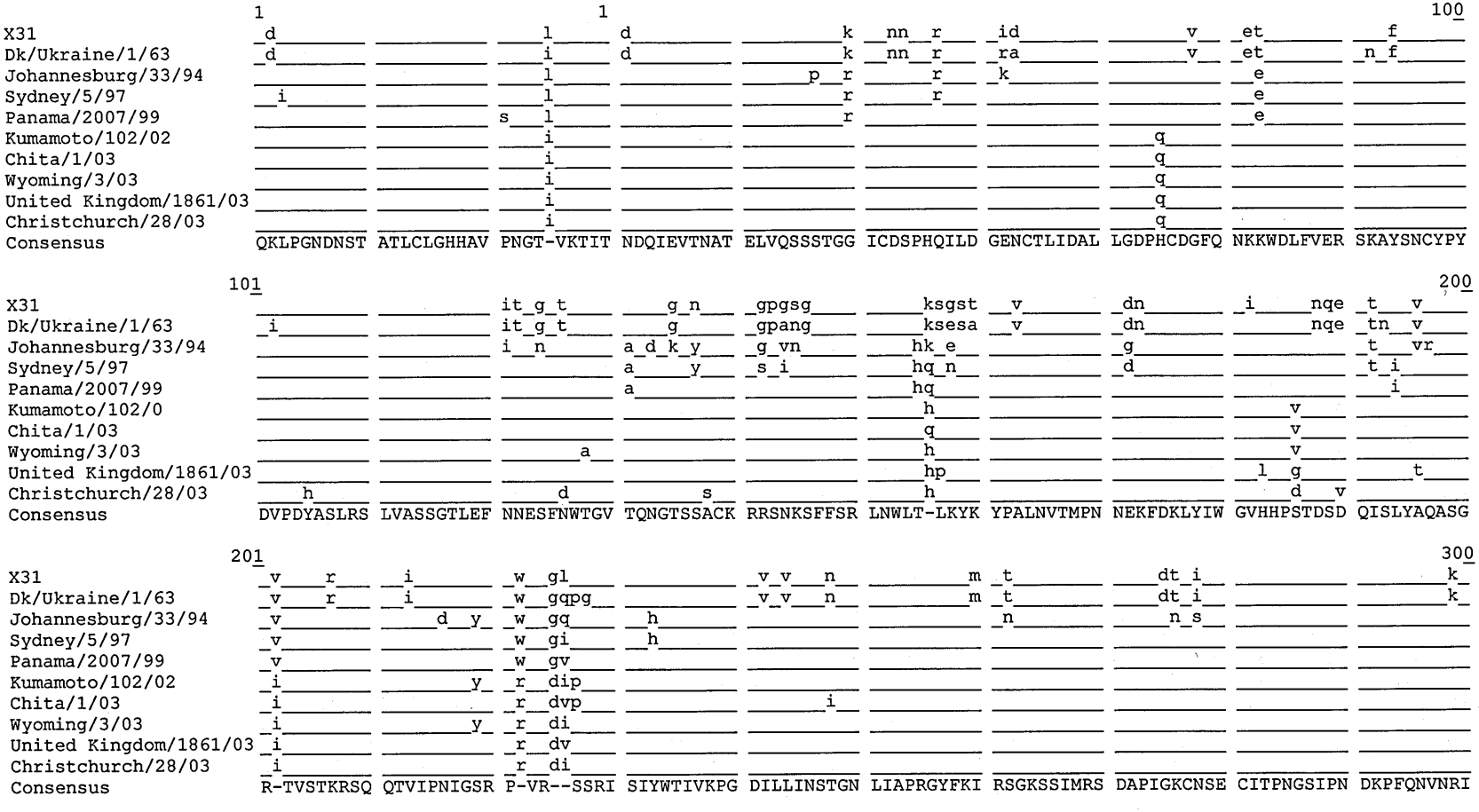
Appendix 3 (continued)

α(2,6)-linkage	α(2,3)-linkage	Reference	Assay/receptor analogues used
Fort Leonard Wood/1/52			
>1mM (>2mM)	0.63mM	(Matrosovich et al., 1993)	K_D, solid phase assay, 3'SL, 6'SLN (6'SL)
256	512	(Rogers and D'Souza, 1989)	HAU, haemagglutination assay (resialyated RBC)
107 %	64%	(Rogers and D'Souza, 1989)	percentage bound compared to native RBC, adsorption assay (resialylated RBC)
Denver/1/57			
0	256	(Rogers and D'Souza, 1989)	HAU, haemagglutination assay (resialyated RBC)
46%	95%	(Rogers and D'Souza, 1989)	percentage bound compared to native RBC, adsorption assay (resialylated RBC)
Brazil/11/78			
16	0	(Ito et al., 1998)	HAU, haemagglutination assay (resialyated RBC)
512	0	(Rogers and D'Souza, 1989)	HAU, haemagglutination assay (resialyated RBC)
109%	18%	(Rogers and D'Souza, 1989)	percentage bound compared to native RBC adsorption assay (resialylated RBC)

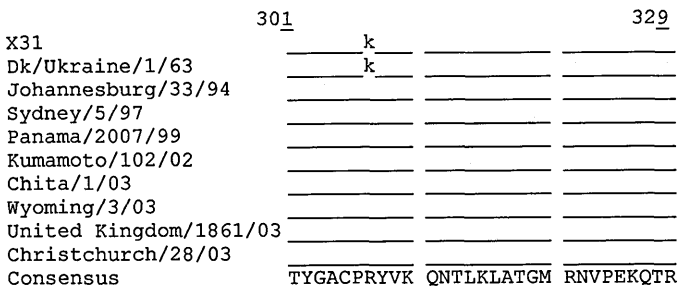
Appendix 3 (continued)

α(2,6)-linkage	α(2,3)-linkage	Reference	Assay/receptor analogues used
Chile/1/83			
0.6mM	0.1mM	(Gambaryan and Matrosovich,1992)	K_D, solid phase assay, 3'SL, 6'SL
64	0	(Ito et al., 1998)	HAU, haemagglutination assay (resialyated RBC)
0.04mM (0.63mM)	0.25mM	(Matrosovich et al., 1993)	K_D, solid phase assay, 3'SL, 6'SLN (6'SL)
256	32	(Rogers and D'Souza, 1989)	K_D, solid phase assay, 3'SL, 6'SL
86%	66%	(Rogers and D'Souza, 1989)	percentage bound compared to native RBC, adsorption assay (resialylated RBC)
Taiwan/1/86			
0.8mM	0.08mM	(Gambaryan and Matrosovich,1992)	K_D, solid phase assay, 3'SL, 6'SL
64	0	(Ito et al., 1998)	HAU, haemagglutination assay (resialyated RBC)
0.2mM (1.25mM)	0.2mM	(Matrosovich et al., 1993)	K_D, solid phase assay, 3'SL, 6'SLN (6'SL)
2048	128	(Rogers and D'Souza, 1989)	HAU, haemagglutination assay (resialyated RBC)
86%	51%	(Rogers and D'Souza, 1989)	percentage bound compared to native RBC, adsorption assay (resialylated RBC)

Appendix 3 (continued)



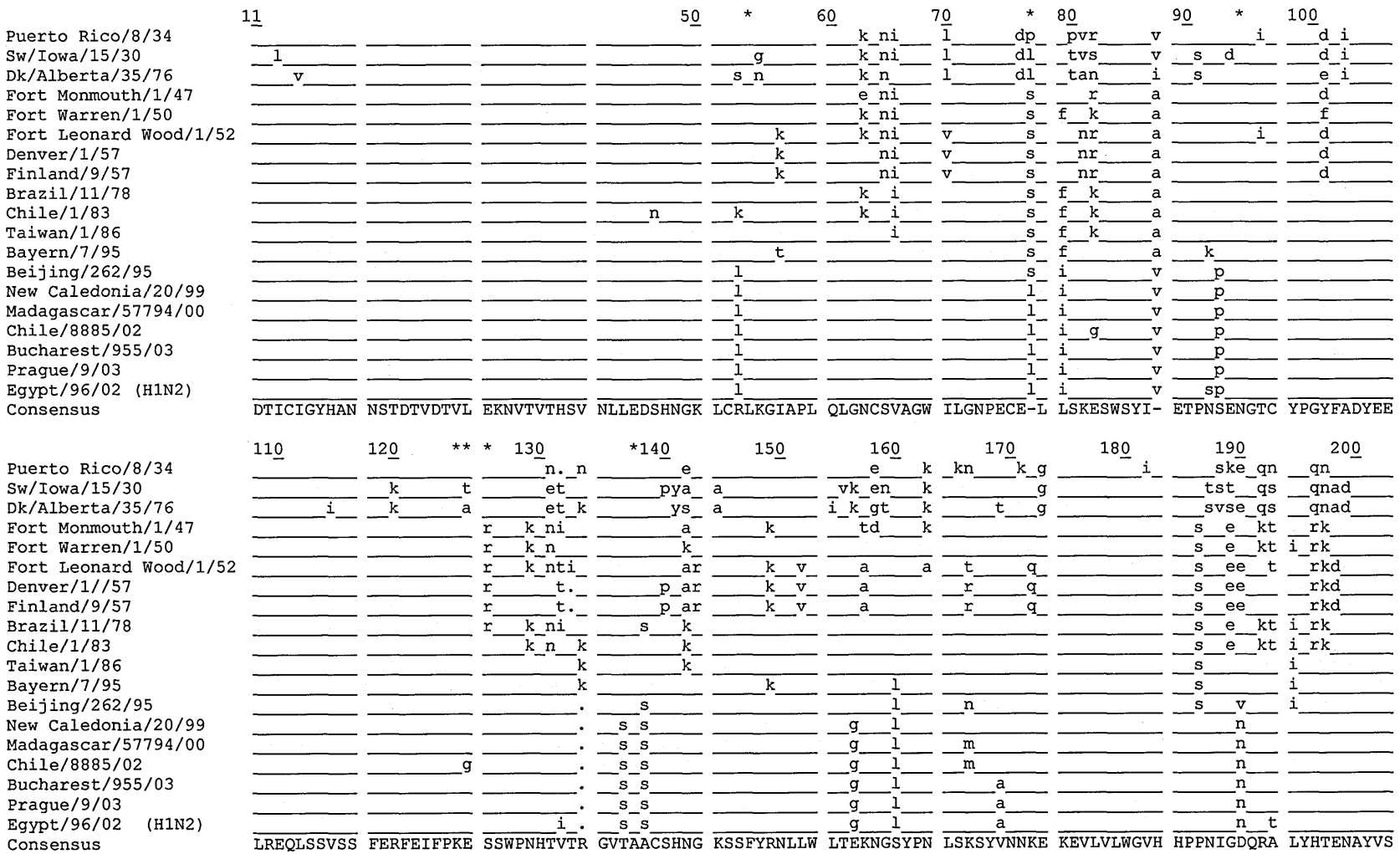
Appendix 4 Amino acid sequence alignment of HA_1 residues of H3 viruses used in this study



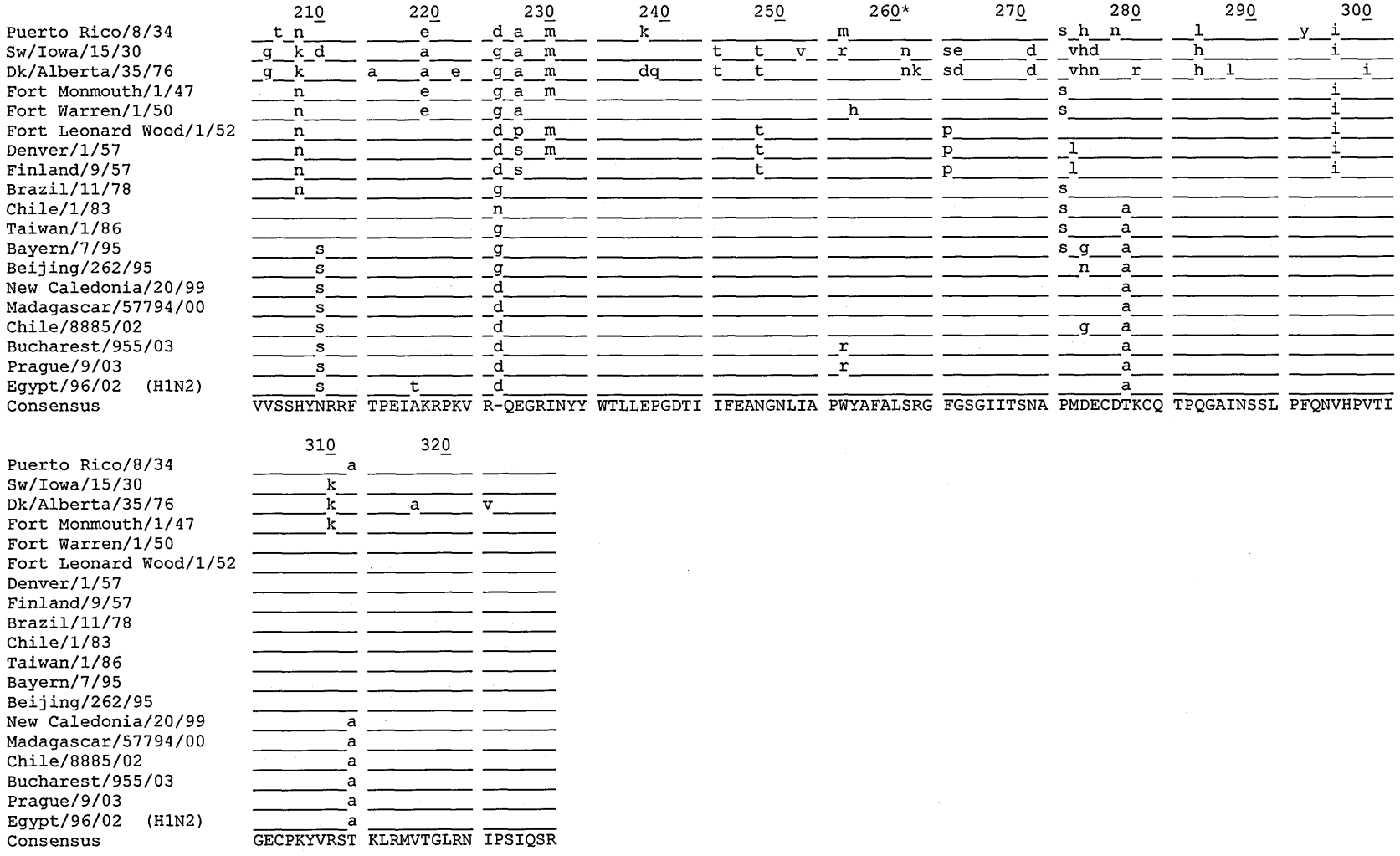
Appendix 4 (continued) Amino acid sequence alignment of HA_1 residues of H3 viruses used in this study

	8	22	38	63	81	122	126	133	144	165	246	276	285	total
X31	x	x	x		x					x			x	6
Dk/Ukraine/1/63	x	x	x							x			x	5
Johannesburg/33/94	x	x	x	x			x			x	x	x	x	9
Sydney/5/97	x	x	x	x		x	x	x		x	x		x	10
Panama/2007/99	x	x	x	x		x	x	x	x	x	x		x	11
Kumamoto/102/02	x	x	x	x		x	x	x	x	x	x		x	11
Chita/1/03	x	x	x	x		x	x	x	x	x			x	10
Wyoming/3/03	x	x	x	x		x		x	x	x	x		x	10
United Kingdom/1861/03	x	x	x	x		x	x	x	x	x	x		x	11
Christchurch/28/03	x	x	x	x		x		x	x	x	x		x	10
	cons	cons	cons							cons			cons	

Appendix 5 Carbohydrate attachment sites of HA_1 residues of H3 viruses used in this study Cons indicates a conserved glycosylation site for the human H3 subtype.



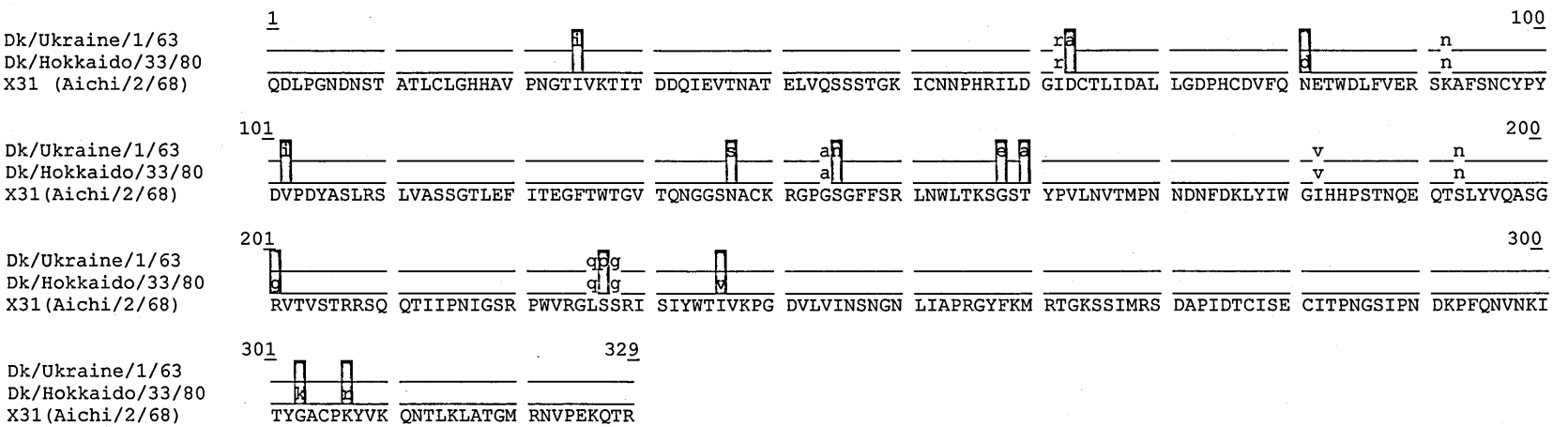
Appendix 6 Sequence alignment of HA_1 residues of H1 viruses used in this study



Appendix 6 (continued) Sequence alignment of HA_1 residues of H1 viruses used in this study

	21	33	63	81	94a	129 or 131	158	163	271	289	total
Puerto Rico/8/34	x	x							x	x	4
Sw/Iowa/15/30	x	x			x					x	4
Dk/Alberta/35/76	x	x			x					x	4
Fort Monmouth/1/47	x	x			x	x			x	x	6
Fort Warren/1/50	x	x			x	x	x	x	x	x	8
Fort Leonard Wood/1/52	x	x		x			x			x	5
Denver/1/57	x	x		x	x	x	x	x		x	8
Finland/9/57	x	x		x	x	x	x	x		x	8
Brazil/11/78	x	x			x	x	x	x	x	x	8
Chile/1/83	x	x			x	x	x	x	x	x	8
Taiwan/1/86	x	x	x		x	x	x	x	x	x	9
Bayern/7/95	x	x	x		x	x		x	x	x	8
Beijing/262/95	x	x	x		x	x		x		x	7
New Caledonia/20/99	x	x	x		x	x		x		x	7
Madagascar/57794/00	x	x	x		x	x		x		x	7
Chile/8885/02	x	x	x		x	x		x		x	7
Bucharest/955/03	x	x	x		x	x		x		x	7
Prague/9/03	x	x	x		x	x		x		x	7
Egypt/96/02 (H1N2)	x	x	x		x	x		x		x	7
	cons	cons								cons	

Appendix 7 Carbohydrate attachment sites of HA_1 residues of H1 viruses used in this study Cons indicates a conserved glycosylation site for the human H1 subtype.



Appendix 8 Amino acid sequence alignment of HA_1 for Dk/Ukraine/1/63, Dk/Hokkaido/33/80 and X31 (Aichi/2/68 HA)
Differences in sequence between Dk/Ukraine/1/63 and Dk/Hokkaido/33/80 are indicated in red.